Experimental Studies on Drug Resistance in Ovarian Cancer

by

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Declaration

I hereby declare that this thesis has been composed by myself and the work is my own except where acknowledged otherwise. Some of the work involved me as a member of a research group and other members are duly acknowledged but only work where I made a substantial contribution is included.

I.P. Hayward

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Abbreviations

AAS	Atomic Absorption Spectroscopy
AraC	Cytosine arabinoside
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BSO	buthionine-S,R-sulfoximine
CBDCA	diammine cyclobutanedicarboxylate platinum (II) (carboplatin)
CCNU	1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
CDNB	1-chloro-2,4-dinitrobenzene
CFE	Colony forming efficiency
CHIP	cis-dichloro-trans dihydroxy-isopropylamine platinum (IV) (iproplatin)
Cisplatinum	cis-diamminedichloroplatinum(II)
DMSO	dimethylsulphoxide
EDTA	ethylenediamine tetra-acetic acid
E.M.	electron microscopy
GSH	reduced glutathione
GSSG	oxidized glutathione
JM40	ethylenediamine-malonato platinum(II)
MESNA	sodium 2-mercaptoethanesulphonate
MOPS	3-[N-morpholino]propanesulfonic acid
NCI	National Cancer Institute, Bethesda, Maryland, U.S.A.
OTZ	2-oxothiazolidine-4-carboxylate
PBS	Dulbecco's phosphate buffered saline (8.0mg/ml NaCl, 0.2mg/ml KCl, 1.15mg/ml Na_2HPO_4, 0.2mg/ml KH_2PO_4)
SEM	standard error of the mean
TCA	trichloroacetic acid
trans-platinum	trans-diamminedichloroplatinum(II)

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ABSTRACT OF THESIS (Regulation 7.9)

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Title of Thesis Experimental studies on drug resistance in ovarian cancer.

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Epithelial ovarian carcinoma is a common and important cancer. Biopsy and ascites samples can be successfully grown in the laboratory enabling this cancer to be studied experimentally. One major problem in this disease is that, while it is a chemosensitive tumour, resistance to current regimens of chemotherapy is a common development. In this project I have established a model system for studying this problem.

Thirty ascites samples and 1 tumour biopsy have been collected and 9 cell lines derived from them. Two cell lines PE/01 and PE/04 derived from ascites from a patient before and after she developed resistance to a regimen of cisplatinum, chlorambucil and 5-fluorouracil have been extensively characterised for cytology, karyotype, antigenicity, and growth characteristics in vitro. PE/04 proved to be 3-fold more resistant to cisplatinum in clonogenic assays but showed little change in sensitivity to chlorambucil and 5-fluorouracil. The results suggest a heterogeneous tumour in vivo with PE/04 cells not being derived from PE/01 cells but rather co-existing with them and being then selected by the chemotherapy. A third cell line from a later ascites from the same patient was also derived together with sub-lines of PE/01 and PE/04 with acquired resistance to cisplatinum in vitro. Cross-resistance to other drugs including platinum analogues of clinical interest and classical alkylating agents has also been assessed. Little cross-resistance to iproplatin (CHIP) and carboplatin (CBDCA) was observed or to unrelated drugs like doxorubicin and vincristine. However resistance to malonato-platinum (JM40) and the modified alkylating agent prednimustine was seen.

Since cisplatinum is the major drug in present chemotherapy of ovarian cancer I investigated mechanisms of resistance to this drug in the above model and looked at a number of aspects known to be significant in resistance to anti-neoplastic drugs. These experiments suggest that:-

1) transport processes are not important in cisplatinum resistance

2) glutathione metabolism plays only a minor role, if at all, in the resistance.

3) the extent of certain types of cisplatinum induced DNA damage is important since the number of DNA interstrand crosslinks was correlated with drug sensitivity. Either reduced DNA damage or increased DNA repair may be the significant factors.

1. Introduction

Ovarian adenocarcinoma is a very important tumour in women and shows itself sensitive to chemotherapy. However, development of resistance has become the major stumbling block in its clinical management. We therefore set out to develop a model to address this problem using human tumour and ascites samples from patients from the Department of Clinical Oncology practice in Edinburgh. The phenomenum of drug resistance has attracted considerable attention and much work has been done by other groups during the period of this project. Here in the introduction I shall discuss literature up until the end of 1983, when this project was begun, as background, and consider developments since then in the relevant chapters and in the general discussion in chapter 7.

1.1 Ovarian Cancer - Present Treatment

Ovarian cancer is the 4th leading cause of cancer deaths in women and the most important and lethal of the gynaecological tumours. Incidence was 16.2/100,000 in Scotland in 1976-1980 with mortality at 13.1/100,000. The total 5 year relative survival rate was 28% and 1 year rate only 49% (Cancer Registration Statistics, Scotland 1971-80). The incidence is highest in postmenopausal women. The vast majority of ovarian tumours (85-90%) are of epithelial histological type with only a minority, primarily in younger women, being sex cord-stromal or

germ-cell tumours (R.C. Young et al, 1982). A number of different histological types have been identified (R.E. Scully, 1977) with serous adenocarcinomas being the most common type followed by undifferentiated carcinomas, endometrial carcinomas, mucinous adenocarcinomas and clear cell tumours (R.C. Young et al, 1982). All appear to be derived from the same multipotential coelomic epithelium.

However, histological type appears not to be an important prognostic factor independent of other factors (R.C. Young et al, 1982). In addition the ovary can be a common site of metastasis from other organs with the most common primary site being the gastrointestinal tract (R.T. Parker et al, 1981). The co-existence of ovarian and uterine adenocarcinoma is also not infrequent with endometroid ovarian carcinoma being associated with endometrium carcinoma in one-third of cases (R.E. Scully, 1970).

Early diagnosis of ovarian cancer continues to be a problem. The search for a suitable marker to allow earlier diagnosis and monitoring of the course of the disease is an active area of research (R.C. Bast et al, 1983). The most frequent presenting symptoms are abdominal pain and extension which are usually indicative of advanced disease (P.J. Di Saia and W.T. Creasman, 1981). The International Federation of Gynecology and Obstetrics has set up a staging classification (O.H. Bears

et al, 1978) with 4 stages which correspond to ovarian (I), pelvic (II), peritoneal or coelomic (III), and metastasizing disease (IV). Stage III and IV represent the majority of patients and the ones with poorest prognosis (J.S. Tobias and C.T. Griffiths, 1976). The other important prognostic variables are histologic grade, i.e. degree of differentiation (R.F. Ozols et al, 1980), and extent of residual disease after surgery (C.T. Griffiths, 1975).

Undoubtedly the cornerstone of therapy is surgery (P.E. Schwartz, 1981) and the initial operation determines the future management of the patient. Accurate staging can only be achieved at this time by abdominal exploration and histopathology. Ideally total hysterectomy, bilateral salpingo-oophorectomy and omentectomy are required and cytoreductive surgery to remove as much tumour as possible is important in advanced disease (C.T. Griffiths et al, 1979). In patients with Stage Ia disease with well differentiated malignancies surgery alone is sufficient for cure (A.J. Dembo et al, 1979a) and it is presumably sufficient in a further subset of patients with long-term survival from surgery alone (C.M. Bagley et al, 1972). However, further therapy is usually necessary with chemotherapy generally being preferred to radiotherapy (M.E. Katz et al, 1981).

The success of additional therapy after initial surgery depends on the existence of residual disease with improved survival in patients with residual tumour nodules of 1-2cms diameter or less regardless of whether chemotherapy (J.T. Wharton et al, 1980) or radiotherapy (A.J. Dembo et al, 1979b) is used. Unfortunately out of approximately 60% of women who present with advanced disease at diagnosis some 75% have such extensive disease that extensive residual disease is left after surgery (R.C. Young et al, 1982). In this group radiotherapy has been less successful and this has led to its diminished use in ovarian cancer.

Chemotherapy

For more than 20 years chemotherapy has relied on the alkylating agents, particularly melphalan, as single agents and their widespread use produced response rates of 35-65% in advanced disease (R.C. Young et al, 1974). However, these responses were limited with median survival of 17-20 months in those responding to chemotherapy versus 6-13 months in non-responders. A number of alkylating agents (melphalan, chlorambucil, cyclophosphamide and thiotepa) show similar activity. Since these response rates were relatively high there have been few trials with other single agents but amongst the most active other drugs are hexamethylmelamine, doxorubicin, 5-fluorouracil, and cisplatinum. Cisplatinum has become the major drug in

combination regimens (see below) and its activity in combination with chlorambucil was soon clearly seen as better than chlorambucil alone (G.H. Barker, et al, 1981a). Results with single agents are summarised in Table I indicating overall response rates in some studies. Various doses and schedules have been used by different workers. Various combinations have also now been reported. The first study, to show improved response rate and improved survival over that of an alkylating agent alone (melphalan) in a randomised controlled trial, was that of Young and his colleagues (R.C. Young et al, 1978) using the HexaCAF combination (hexamethylmelamine, cyclophosphamide, methotrexate and 5-fluorouracil). This achieved a 75% response rate versus 54% for melphalan and gave an increased medium survival of 29 months versus 17 months. This study also illustrated the need to stratify for important prognostic factors to convincingly demonstrate improvements with combination chemotherapy. The improvements with this regimen could not be confirmed by two other groups (J. Carmo-Pereira et al, 1981; J.F.G. Sturgeon et al, 1980) but were seen by a third group (J.P. Neijt et al, 1980) although with a lower response rate.

However, further testing of this and other combinations was rapidly overtaken by interest in combinations including cisplatinum. The NCI group went on to study the CHex-UP (cyclophosphamide, hexamethylmelamine, 5-

TABLE 1

SINGLE AGENTS ACTIVE IN ADVANCED OVARIAN CANCER

Drug	No. of Patients	Response rate (%)	Reference
Alkylating agents			
Melphalan	541	47	J.S. Tobias and C.T. Griffiths, 1976
Chlorambucil	388	51	
Thiotepa	337	48	
Cyclophosphamide	335	43	
Cyclophosphamide (high dose)	36	61	
Non-alkylating agents			
Hexamethylmelamine	54	32	J.T. Wharton et al,1979
Doxorubicin	34	27	J.P. Smith, 1978
5-fluorouracil	81	32	R.C. Young et al, 1974
Cis-platinum	18	22	H.W. Bruckner et al, 1981a
Cis-platinum	22	50	D.M. Gershenson et al, 1981
Cis-platinum	21	52	E. Wiltshaw et al, 1983
Doxorubicin vs	34	27	J.P. Smith, 1978
Hexamethylmelamine vs	33	31	
Melphalan	33	30	

fluorouracil and cisplatinum) regimen (R.C. Young et al, 1979). Other similar multiple drug combinations (e.g. CHAP - cyclophosphamide, hexamethylmelamine, doxorubicin and cisplatinum and PAC - cisplatinum, doxorubicin and cyclophosphamide) have been tried by others. The results of various combinations are summarised in Table II. Responses to cisplatinum containing combination chemotherapy can be seen to be significantly higher than responses to conventional alkylating agent therapy.

Variations between studies are often due to different proportions of patient groups with patients with minimal residual disease having a much better chance of achieving a complete response. Various doses and schedules of drugs have been employed. Although response rates have improved, this has had a relatively minor effect on median survival times. In some studies the difference in survival has been minimised by cross-over from the alkylating agent arm to the combination regimen upon progression. Complete responses are only a proportion of the total number of responses and more recently pathologically proven complete response rates, as seen by second look laparotomy are only about half the clinical complete response rate. Responders to therapy do have improved survival but it is the pathologically complete responders who have a good chance of long term survival. Thus the proportion of long term survivors is still

TABLE II
COMBINATION CHEMOTHERAPY IN ADVANCED OVARIAN CANCER

Regimen	No. of Patients	Response Rate %	Complete Response Rate (%)	Time to Progression (months)	Median Survival (months)	Reference
Melphalan vs	37	54	16		17	R.C. Young
HexaCAF*	40	75	33		29	et al (1978)
Cyclophosphamide vs	29	62	24	10	11	J. Carmo-Pereira
HexaCAF	28	36	14	9	10	et al (1981)
Melphalan vs	38		13		NSD	J.F.G. Sturgeon
HexaCAF vs	37		19		NSD	et al (1982)
CAP	40		30		NSD	
				(but crossover from Melphan to CAP)		
Melphalan vs	72	40	10		10.7	
Melphalan + Doxorubicin	70	63	30		16.8+	C. Trope (1981)
Cyclophosphamide vs	35 (14)	31	0	6 (12)	12 (17)	J.H. Edmonson
Cyclophosphamide + Doxorubicin	36 (13)	36	6	6 (15)	12 (42+)	et al (1979)
patients with bulky residual disease (minimal residual disease in brackets)						
Melphalan vs	64	37	20	5.0	12.3	G.A. Omura et al (1983)
Melphalan + Hexamethylmelamine vs	97	52	28	6.6	13.5	
Cyclophosphamide + Doxorubicin	72	49	32	6.3	14.2	

TABLE II (contd)
COMBINATION CHEMOTHERAPY IN ADVANCED OVARIAN CANCER

Regimen	No. of Patients	Response Rate %	Complete Response Rate (%)	Time to Progression (months)	Median Survival (months)	Reference
Chlorambucil vs	19	23		7	17	D.R. Bell et al (1982)
Cyclophosphamide + Cis-platinum	17	69		13	17	
Cyclophosphamide vs	21		14 (5)	7	17	D.G. Decker et al (1982)
Cyclophosphamide + Cis-platinum	21		48 (24)	28	44	
	(brackets - pathological complete response rate PCR)					
Chlorambucil vs	16	25	0 (PCR)			G.H. Barker et al (1981a)
Chlorambucil + Cis-platinum vs	46	52	28			
Chlorambucil + Cis-platinum + Doxorubicin	39	54	28			
Cyclophosphamide + Doxorubicin vs	101	46	20 (5)	9.5		G.A. Omura et al (1982)
Cyclophosphamide + Doxorubicin + Cis-platinum	91	71	44 (20)	15.0		
Cis-platinum vs	18	22	6	9	20	H.W. Bruckner
Cis-platinum + Doxorubicin vs	18	67	33	15	19	et al (1981a)
Thiotepa + Methotrexate	17	29	6	3	11	

TABLE II (contd)
COMBINATION CHEMOTHERAPY IN ADVANCED OVARIAN CANCER

Regimen	No. of Patients	Response Rate %	Complete Response Rate (%)	Time to Progression (months)	Median Survival (months)	Reference
Chlorambucil vs	27	26	22 (PCR)		17	C.J. Williams
PAC	27	72	26		17	et al (1983)
PAC (I) vs	31	75	39	22.8	23.5	C.E. Ehrlich
PAC (V)	25	88	44	15.7	27.5	et al (1983)
CHex-UP	51	75	41 (20)		18	R.C. Young et al (1981)
Melphalan vs	123	44	20	8.5	17	S.E. Vogl
CHAD	123	63	41	13.9	19	et al (1982)
Cis-platinum + Doxorubicin vs	20	78	43	18	18	H.W. Bruckner
CHAP	37	83	57	25	25	et al (1983)
Hexa-CAF vs	94	50	17		20	J.P. Neijt
CHAP-5	92	79	30		31	et al (1983)

*Hexa-CAF - Hexamethylmelamine, Cyclophosphamide, Methotrexate and 5-Fluorouracil
CAP, PAC - Cyclophosphamide, Doxorubicin and Cis-platinum
CHex-UP - Cyclophosphamide, Hexamethylmelamine, 5-Fluorouracil and Cis-platinum
CHAD, CHAP, CHAP-5 - Cyclophosphamide, Hexamethylmelamine, Doxorubicin and Cis-platinum

depressingly low even in the face of aggressive combination chemotherapy with its associated considerable toxicity. Indeed its use has been questioned particularly in patients with bulky residual disease (C.J. Williams et al, 1983) because of its toxicity and minimal effect on these patients' survival.

Clinical Resistance

As can be seen from the above, many tumours are responsive to therapy but a subpopulation of cells is either resistant or rapidly acquires resistance causing the regrowth of the tumour and the subsequent short survival times of patients.

Once a patient has relapsed after initial therapy the response to second line therapy has been poor with responses generally only of short duration (up to about 6 months) [R.C. Young et al, 1982]. Most of the drugs in current use were first shown to be active against ovarian cancer by demonstrating this limited second line activity after the failure of initial alkylating agent therapy, and this subsequently led to their evaluation for initial regimens as discussed above, but it has had little impact when initial treatment fails. Cis-platinum is perhaps the most active second line agent, first reported in 1976 and showing response rates of around 30% (E. Wiltshaw et al, 1976; H.W. Bruckner et al 1978). Its use has even been

reported in a third line capacity with some efficacy (M.S. Piver et al, 1980). Hexamethylmelamine has also shown significant activity as a second line agent with a response rate of approximately 16% (G.A. Omura et al, 1981). Doxorubicin has less activity in this capacity (S.M. Hubbard et al, 1978). Some of the combination regimens have also been used with responses of 40-60% being reported for some cis-platinum containing regimens as shown in Table III. However, since these regimens achieve higher initial remission rates than alkylating agents they have not continued in second line use after alkylating agent therapy. The present goal of therapy is the achievement of pathological complete remissions with the initial modality of treatment and it is these patients who have a high chance of long term survival (P.E. Schwartz et al, 1980).

Four potential methods of overcoming clinical drug resistance are currently being pursued:-

(1) The use of high dose cis-platinum (greater than 100mg/m^2) is being assessed and a clinically important dose-response relationship has been demonstrated with responses in patients previously treated with lower dose cis-platinum having been achieved (G.H. Barker et al, 1981b).

(2) Intraperitoneal administration of chemotherapy in an attempt to increase the concentration of drug

TABLE III
CHEMOTHERAPY AFTER ALKYLATING AGENT FAILURE

Regimen	No. of Patients	Response Rate %	Complete Response Rate	Time to Progression	Median Survival	Reference
Cis-platinum						
30mg/m^2	52	33	6	4		E. Wiltshaw
100mg/m^2	30	52	16	7		et al (1979)
Hexamethylmelamine	49	16	2	4		G.A. Omura et al (1981)
Doxorubicin	18	5			3	S.M. Hubbard et al (1978)
Cis-platinum + Hexamethylmelamine	40	57	20	8		T. Davis et al (1980)
Cyclophosphamide + Doxorubicin + Cis-platinum	24	50	8		7	H.W. Bruckner et al (1978b)
Hexamethylmelamine + Doxorubicin + Cis-platinum	27	67	18	7	10	S.E. Vogl et al (1979)
CHAP	35	49	20	6		R. Kane et al (1979)

TABLE III (contd)
CHEMOTHERAPY AFTER ALKYLATING AGENT FAILURE

Regimen	No. of Patients	Response Rate %	Complete Response Rate	Time to Progression	Median Survival	Reference
Cis-platinum + Doxorubicin vs	20	25		5.3	6.9	J.P. Neijt et al (1982)
Cis-platinum + Doxorubicin + Hexamethylmelamine	21	19		4.1	5.3	
	(and responders survival no longer than non-responders)					
Cis-platinum vs	24	25			7	H.W. Bruckner et al (1981b)
Cis-platinum + Doxorubicin vs	43	36			7	
Cis-platinum + Doxorubicin + Cyclophosphamide vs	43	51			7	
CHAP	18	49	22		7	

For abbreviations see Table II

reaching the tumour is being used (R.B. Jones et al, 1978). Since even advanced ovarian carcinoma usually remains confined to the abdominal cavity high local concentrations of drugs achieved in the peritoneum without systemic toxicity (J.L. Speyer et al, 1981) could be very advantageous. This technique has been tried with doxorubicin (R.F. Ozols et al, 1982), 5-fluorouracil (J.L. Speyer et al, 1980) and cis-platinum (R.G. Pretorius et al, 1983). With intraperitoneal cis-platinum intravenous thiosulphate has been used to protect against cis-platinum toxicity and preliminary results indicate responses are achievable in patients with extensive prior therapy (S.B. Howell et al, 1983).

(3) In vitro assays to predict to which drug(s) a particular patient would be responsive have been attempted (D.S. Alberts et al, 1981). While reasonable correlations have been achieved with the clonogenic assay (D.D. Von Hoff et al, 1983) many problems remain (P. Selby et al, 1983) and unfortunately many samples have not grown in vitro or been resistant to the drugs tested. The assay is still experimental and is discussed in the next section.

(4) New drugs are being sought which might have improved

efficacy against ovarian cancer as the lack of sufficiently active drugs is obviously one of the major problems. Currently cis-platinum analogues are attracting the most interest particularly because of their reduced nephrotoxocity compared with cis-platinum but with similar response rates (E. Wiltshaw et al, 1983). However, there is some suggestion that carboplatin (cis-diammine-1,1-cyclobutanedi-carboxylate platinum II) may have some activity in cis-platinum resistant ovarian tumours (B.D. Evans et al, 1983).

Perhaps a fifth area of research should be mentioned here, namely the search for techniques to allow earlier detection of the disease since earlier stage tumours show much better and more durable responses to current therapy. Unfortunately no ideal tumour marker has yet emerged for this purpose and current research is concentrating on possible tumour associated antigens using monoclonal antibodies (M. Bhattacharya et al, 1982; R.C. Bast et al, 1983).

1.2 Ovarian cancer - laboratory studies

Experimental work with tissue of human origin has lagged well behind that from animals especially rodents. Tissue from human ovarian tumours received relatively little attention up until recently and in vitro growth of human

tumour cells was regarded as a rare and unusual event. Indeed in 1975 Fogh and Tempe in preparing an inventory of all published human tumour cell lines (J. Fogh et al, 1975) found only 4 human ovarian tumour lines in the literature. However, work by Ioachim and his colleagues (H.L. Ioachim et al, 1974) showed that at least short term culture could often be achieved with 46 of 62 ovarian tumour samples showing initial growth and 35 of these being cultured for more than 30 days. Morphological, cytological and immunological features could be analysed.

Clonogenic assays

Interest in culture of human ovarian carcinoma cells was stimulated by the development of a tumour cloning assay in agar by Hamburger and Salmon in which ovarian carcinoma cells taken from ascites samples grew well compared with other tumour types (A.W. Hamburger and S.E. Salmon, 1977). It had been shown as long ago as 1964 that tumour cells grew selectively in agar compared with normal cells (I. MacPherson et al, 1964). Further work showed tumour colony growth from both effusions and biopsies from 85% of 31 ovarian cancer patients (A.W. Hamburger et al, 1978). At the same time a similar technique was developed by Courtenay and her colleagues (V.D. Courtenay and J. Mills, 1978) and again ovarian tumours were among the best for growth in the agar cultures (V.D. Courtenay et al, 1978). These assays separate biopsies or effusions into single

cell suspensions by either mechanical or enzymic disaggregation and assess the cloning efficacy by the number of colonies which grow up as clones from the single cell suspension immobilised in agar. The major use of these techniques has been in determining the sensitivity of these tumour specimens to various antineoplastic drugs with the idea of first correlating in vitro sensitivity with the patient´s clinical sensitivity and then hoping to be able to predict clinical sensitivity and so help in the clinicians choice of therapy (D.S. Alberts et al, 1980). Although this goal has not yet been achieved (D.D. Von Hoff et al, 1983) correlations with patients clinical responses of approximately 95% for drug resistance and 60-70% for drug sensitivity have been reported (B.I. Sikic et al, 1981). The assay has been criticised for various methodological problems (F.R. Mackintosh et al, 1981; I. Bertoncello et al, 1982; M.V. Agrez et al, 1982; P. Selby et al, 1983) and is being refined by various groups. Although around 80% of ovarian tumour samples will grow in the Hamburger/Salmon assay with ascites samples growing more frequently than solid tumour samples (D.D. Von Hoff et al, 1981) plating efficiencies of 0.001 to 1% only are achieved (I. Bertoncello et al, 1982) with 500,000 cells per 35mm dish being commonly plated. This means even a low number of cell aggregates in the original preparation can be a problem in assessing colony formation at the end of the assay (M.V. Agez et al, 1982). Abortive

colonies are also difficult to assess. In addition the cell survival curves obtained have often shown unexpected plateaus with increasing dose unlike most in vitro experience suggesting this may be artifactual (P. Selby et al, 1983). The in vitro/in vivo correlations have been based on doses of drugs in vitro based on peak plasma concentrations achievable in vivo (T.E. Moon et al, 1981) with 1/10th peak plasma concentration often being used (D.D. Von Hoff et al, 1983). This drug concentration used in the assay plus the cut-off point of <30% survival for assessing sensitivity have been thought to be somewhat arbitrary by some authors (P. Selby et al, 1983). Obviously improvements in this assay are necessary but the clinical correlations have been sufficient to stimulate continued interest. Unfortunately the majority of samples have shown drug resistance in vitro and in the minority showing sensitivity (approximately 20%) the correlation with the clinical outcome has not been as impressive (T.E. Moon et al, 1981; D.D. Von Hoff et al, 1983). Thus the number of patients who could be helped is very limited at this stage (T.J. Williams et al, 1983). While the Courtenay assay has not been used as extensively it warrants further use due to the increased plating efficiency seen with this procedure, thought to be due to the low oxygen concentration used (5% vs 20% in the Hamburger/Salmon assay) and the presence of rat red blood cells (K.M. Tveit et al, 1981).

This type of methodology has also been used by one group in attempting to address the question of what constitutes the stem cell population of a tumour (W.J. Mackillop et al, 1983a). Obviously this population is critical to the continued growth of a tumour and it would be important to know if the low percentage of clonogenic cells observed in vitro were representative of or represented this population. While culture conditions are undoubtedly sub-optimal the cells with self-renewal capacity seem to be a subpopulation of the clonogenic cells (R.N. Buick et al, 1981) as assessed by secondary plating efficiency. Cellular heterogeneity could be observed in ascites samples and different cell populations (non-proliferative, differentiated cells of high density versus proliferative and clonogenic cells of lower density) could be separated by density gradient fractionation (W.J. Mackillop et al, 1981). In a series of 7 ascites samples collected from one patient over a 9 months period changes in the density distribution of the cell population could be observed with an increase in the number of clonogenic cells but particularly an increase in the secondary colony plating efficiency (W.J. Mackillop et al, 1983b).

Cell characterisations

Other studies with human ovarian carcinoma cells have described the different cytological features of ovarian carcinomas. This includes the different histological

types and the different grades of differentiation, the presence of various antigens and a number of studies on chromosomal abnormalities. Ioachim concluded that ovarian carcinomas in short-term culture preserve most of their salient features as observed by light microscopy and electron microscopy (H.L. Ioachim et al, 1974). The histologic and cytologic features of the common epithelial ovarian tumours were recently reviewed by Fenoglio (C.M. Fenoglio, 1980). Ioachim and others also produced heterologous antisera to ovarian carcinoma extracts (H.L. Ioachim et al, 1975; J.J. Barlow et al, 1975). Since these antisera require extensive absorption to remove reactivities against normal host tissues the reactivity has recently been compared with that of the monoclonal antibody approach (R. Berkowitz et al, 1983). Most recent attention has focussed on producing monoclonal antibodies (R.C. Bast et al, 1981; M. Bhattacharya et al, 1982) in the search for a specific marker to monitor this disease. Various studies have looked at other antigens including markers such as carcinoembryonic antigen (W.G. Haije, 1982) and also hormone receptors including those for oestrogen and progesterone (A. Bergqvist et al, 1981). Cytogenetic studies have shown a considerable number of abnormal chromosomes of various sorts with cells ranging from hypodiploid to tetraploid (C.D. Olinici et al, 1973). Wake and colleagues have suggested a 6:14 translocation may be consistently involved in ovarian cancer (N. Wake et

al, 1980). Others have found similar deletions in chromosome 6 but alterations in chromosomes 1 and 3 have also been quite common (J.M. Trent et al, 1981).

Ovarian cell lines

A number of human ovarian cell lines have been reported since the mid 1970s. In the earlier reports simply cellular morphology, growth rates and some karyotype information were reported (J. Fogh et al, 1975; P.J. DiSaia et al, 1975; R.S. Freedman et al, 1978; G.A. Sinna et al, 1979) with epithelial characteristics and aneuploidy being shown. Recent reports have given more detailed characterization. Woods and her co-workers compared 4 cell lines for isozyme patterns, hormone production and hormone receptor proteins and showed detailed karyotypes (L.K. Woods et al, 1979). Simon and his colleagues showed a higher success rate in establishing cell lines and investigated the effects on growth of various hormones (W.E. Simon et al, 1979; W.E. Simon et al, 1983). Welander used irradiated macrophage feeder layers to assist the initial growth of 25 cell lines beyond 5 passages (C.E. Welander et al, 1982) with greater success for metastatic nodules than solid tumours. Van Haaften-Day established 14 cell lines and 6 xenografts in nude mice from samples from 10 patients and showed changes in DNA content towards aneuploidy and a decrease in hormone receptors in early passages in culture (C. Van

Haaften-Day et al, 1983). Other recent attempts to establish xenografts have shown greater success from solid tumours than ascites (S. Kullander et al, 1978; G.G. Steel et al, 1983). A group at the N.C.I. has also recently shown interest in establishing human ovarian cell lines to investigate drug resistance (T.C. Hamilton et al, 1983a,b). Obviously now that growth of ovarian carcinoma cells in vitro has been demonstrated, various groups are using them to investigate their particular interests. There are some cell lines without published characterisations being used in this way, for example to test monoclonal antibodies (R.C. Bast et al, 1981) and to demonstrate the presence of oncogenes (A. Eva et al, 1982) and the general area of the culture of human ovarian cancer cells is likely to continue to expand.

1.3 Drug resistance studies

Drug resistance is undoubtedly a major clinical problem and various studies have attempted to understand the patterns of resistance between different drugs and to elucidate mechanisms of resistance. Much of this work has been done with mouse tumours and in vitro with various mammalian cell lines and only recently has the importance of using human cell material been recognized.

Murine tumour studies

A very major contribution to anti-cancer drug resistance

studies has been that of Schabel, Skipper and their colleagues mainly using the L1210 and P388 murine leukaemias, and various sublines selected for mutation to drug resistance, to plot the rate of appearance of resistance and to analyse the subsequent relative sensitivity to the drugs. In 1972 they produced a reference chart of cross resistance or sensitivity amongst 27 agents of clinical interest (H.E. Skipper et al, 1972) in which cross resistance between certain closely related drugs was demonstrated but continued sensitivity to unrelated drugs was more often the case. Detailed work with the alkylating agents has shown that cells selected for resistance to one alkylating agent may remain fully sensitive to some other alkylating agents and still show some sensitivity to others (F.M. Schabel et al 1978). Various factors have been investigated including the total body tumour cell burden, growth and regrowth rates of sensitive and resistance cells, ratios of sensitive to resistant cells, drug dose levels, intervals between doses, duration of treatment and choice and sequence of drugs (H.E. Skipper et al 1978). The rate of spontaneous mutation to resistance appears to vary markedly in these tumours for various drugs being highest for the mitotic inhibitors like Vincristine and lowest for alkylating agents like Cyclophosphamide. Treatment failures occur with large tumour burdens (greater than approximately 10^7 cells for single agents) due to overgrowth of drug-

resistant tumour cells after initial responses. Changing to a new drug when the tumour burden reaches its nadir can result in therapeutic gain and even cure (F.M. Schabel et al 1980). Sequential rather than combined drug protocols seemed to give better results usually because full doses of the drugs could be employed. The second drug or drugs could be selected on the basis of profiles of cross-resistance. Resistant cells which overgrow a tumour can also sometimes show collateral sensitivity to other drugs. Cross resistance patterns for over 100 drugs have now been established in these models (F.M. Schabel et al 1983).

Theoretical models

The mathematical model of Norton and Simon has added to our understanding of the effects of tumour size and growth rate on the effectiveness of therapy (L. Norton and R. Simon 1977) since most clinical tumours show gompertzian growth, where growth is slowed down in large tumours, rather than the exponential growth in some simple experimental systems. The model suggests that resistance can sometimes be due to the timing, dosage and duration of the initial therapy as can be seen from tumours which regrow after successful initial therapy but then still respond to that therapy and the model can successfully fit various experimental data.

However, the overgrowth of a tumour by truly drug-

resistant cells has led to another influential mathematical model, that of Goldie and Coldman, who modelled the spontaneous mutation rate of a tumour to suggest when and how fast drug resistant cells would be generated (J.H. Goldie and A.J. Goldman 1979). The likelihood of finding any cells with resistance to a specific drug is related to the size of the tumour and the mutation frequency and in this model the drug acts only as a selecting agent rather than a mutating agent in its own right. The model is based on that of Luria and Delbruck who showed resistance to viral infection in bacteria arose by random spontaneous mutations which occurred at a fixed frequency (S.E. Luria and M. Delbruck 1943). Their fluctuation test, which has now been applied to resistance to chemotherapeutic agents in mammalian cells, shows that since the mutations occur randomly the number of resistant cells in each of a number of parallel subclonal cultures of a certain size will fluctuate to a large extent. Thus for a tumour of a certain size a probability value for the number of resistant mutant cells to be expected can be given based on the mutation rate but the resistant cell(s) could have arisen at any time in the tumour´s progression. Therefore one prediction of the model is that chemotherapy should be begun as early as possible but there will be a variable response between similar sized tumours of the same type in different patients. Secondly it suggests that alternating non-cross-resistant chemotherapy between

two active regimens would usually be the optimum treatment to maximise the chance of avoiding the development of doubly resistant cells and combination chemotherapy would be superior to single agent treatment (J.H. Goldie et al 1982). The model also predicts that slower growing tumours will be more resistant since there will have been many more division cycles of the stem cells for the population as a whole to reach any given size (J.H. Goldie et al 1983). Basically the strategy suggested is to limit to a minimum the time that any resistant subtype has to grow unimpeded by therapy. Various experimental data, particularly with mammalian cell lines in culture, support this concept of the genetic basis of resistance, with mutation frequencies of 10^{-5} to 10^{-7} (V. Ling 1982). Selection of a pre-existing minor clone of cells has been demonstrated (J.T. Isaacs et al 1981) and heterogeneity in tumour subpopulations has been observed by many groups (T. Tsuruo et al 1981).

Other concepts

Other concepts which may also explain some drug resistance include the pharmacology of drugs in reaching the tumour, including factors such as its vascularization, and also the kinetic status of the tumour with cycling cells being preferentially killed. A steep dose response curve is observed for many agents and so where extra toxicity can be avoided, for example by regional chemotherapy, then an

increased response can be achieved by increasing the dose (E. Frei et al 1980). The pharmacokinetics of different anti-neoplastic drugs concerning their absorption, distribution, metabolism including active metabolites, and excretion (M.G. Donnelli et al 1977; E. Mihich 1980) is an increasing area of study due to the recent use of more sensitive and specific assay methods (C. Erichman et al 1980) particularly high pressure liquid chromatography, and can explain apparent tumour resistance (R.L. Dedrick et al 1975) where not enough active drug reaches the tumour. The presence of hypoxic cells in tumours, often due to poor vascularization (I.F. Tannock 1968) has also been demonstrated and can protect cells from both radiotherapy and chemotherapy (K.A. Kennedy et al 1980). In vitro studies with multicellular spheroids have also shown central hypoxia and also poor penetration of some drugs such as Doxorubicin (R.E. Durand 1981). Most of the presently available drugs are more toxic against cycling cells (W.R. Bruce et al 1966; B. Drewinko et al 1981) and so the presence of non-proliferating or G_o cells in a tumour could limit its response to therapy (V.T. DeVita 1971). Many of the commonly used drugs have also been shown to be more toxic in a particular phase of the cell cycle in tumour cell lines in tissue culture (F. Mauro et al 1970). This has led to a large body of data especially with the L1210 murine leukaemia involving the scheduling of various drugs to take account of these factors (I.

Tannock 1978) but it has been much more difficult to translate these results to scheduling in man and kinetic data on human tumours are much more limited (I. Tannock 1978; B.T. Hill 1978).

1.3.1 Biochemical mechanisms of resistance

Most of the above concepts have been elucidated in animal tumour models or cell lines in vitro. The studies of the mechanisms by which truly resistant tumour cells avoid drug toxicity has largely been determined in murine or chinese hamster cell lines. The mechanisms so far identified cover the whole ambit of cellular activities including defective drug transport either influx or efflux, defective metabolism to active species, increased drug inactivation, altered pools of competing normal metabolites and enzymes, altered target proteins and increased quantity of target protein via gene amplification, and altered DNA repair.

Methotrexate resistance

Methotrexate is the most studied drug regarding its mechanism of action and acquired resistance to it. It acts as an inhibitor of the enzyme dihydrofolate reductase required in the de novo synthesis of thymidine and purines. Resistance at almost every step of its metabolism has been shown. In the 1960s defective transport (D. Kessel et al 1965) and increased levels of

the target enzyme dihydrofolate reductase (J.L. Biedler et al 1963) were observed. Later altered dihydrofolate reductases with decreased affinity for drug (W.F. Flintoff et al 1976) were recognised and more recently reduced metabolism of methotrexate to its polyglutamate forms (K.H. Cowan et al 1983) or the efficiency of purine salvage pathways (G.P. Browman et al 1981) have been indicated. It has been suggested that the transport and increase in target enzyme mechanisms are the most important (F.M. Sirotnak et al 1981), although more than one of the above mechanisms can be found together (D.A. Haber et al 1981). Detailed analyses have shown different types of transport defect including both a reduced Vmax and increased Km for carrier mediated methotrexate influx (B.T. Hill et al 1979; F.M. Sirotnak et al 1981). The increase in the amount of target enzyme can occur by increased synthesis and delayed turnover (R.C. Jackson et al 1973) but the major mechanism has been shown to be via gene amplification (R.T. Schimke et al 1978) with stable resistance being associated with specific expansions of the chromosome known as homogeneously staining regions, and unstable resistance with extra-chromosomal elements called double minute chromosomes (R.J. Kaufman et al 1981). The mechanism of gene amplification is thought to be by extra rounds of DNA replication and may represent an important general mechanism for altering gene activity (A. Varshavsky 1981). Recent DNA-mediated genetic transfer

techniques have allowed the transfer of DNA coding for the dihydrofolate reductase gene thus confering methotrexate resistance on the recipient cell (K. O´Hare et al, 1981). Efforts have also turned towards the synthesis of different folate antagonists to circumvent methotrexate resistance either at the membrane (T. Ohnoshi et al 1982) or enzyme (S. Dedhar et al, 1983) level. In the clinical setting high dose methotrexate with leucovorin rescue has been used (J.R. Bertino 1977).

Pleiotropic resistance

The importance of gene amplification has also been suggested in the so-called pleiotropic resistance phenotype (J.L. Biedler et al 1983). This phenotype is best characterised in chinese hamster cell lines and cross resistance to various drugs principally the anthracyclines, vinca alkaloids, colchicine and actinomycin D is observed (N.T. Bech-Hansen et al 1976). This cross resistance appears to be due to reduced drug transport with membrane permeability being implicated (V. Ling et al 1973) and the presence of an approximately 170,000 MW cell surface glycoprotein (R.L. Juliano et al 1976) being closely correlated with drug resistance. Expression of this glycoprotein was co-transferred with drug resistance by DNA-mediated gene transfer from the resistant mutants (P.G. Debenham et al 1982). In mouse leukaemia models increased drug efflux has been suggested

to be the critical factor (M. Inaba et al 1981) and the blocking of this process by calcium channel blockers and calmodulin inhibitors has increased sensitivity again (T. Tsuruo 1983). This phenotype has attracted considerable attention since the cross-resistance is acquired even when different drugs are used to select resistance and so it could affect many combination chemotherapy regimens (V. Ling et al 1983).

Other agents

Other agents have shown different mechanisms of resistance. For antimetabolites like 5-fluorouracil and cytosine arabinoside, which act through the appropriate nucleoside metabolism pathways, the activities of various enzymes and levels of normal metabolites in these pathways are often the critical factors, making for a complex picture of affected metabolism (B. Ardalan et al 1980).

For the alkylating agents drug inactivation and DNA repair can play a role. The cellular toxicity of the phenylalanine mustards has been recently reviewed by Vistica looking at the above factors and also emphasising some of the pitfalls in experimental methodologies such as media composition in tissue culture which can affect the results (D.T. Vistica 1983). Most work has been done with melphalan, which is actively transported into cells by amino acid carrier systems particularly that for leucine (A. Begleiter et al 1979) and resistance at this transport

level has been demonstrated in L1210 cells (W.R. Redwood et al 1980). Some of the other alkylating agents are apparently carrier independent (J.E. Byfield et al 1981). Intracellular sulphydryl compounds capable of participating in alkylating agent inactivation have also been shown to play a role. Melphalan resistance has been related to levels of glutathione, the principal non-protein thiol in cells (K. Suzukake et al 1983) and reversal to drug sensitivity correlated with glutathione depletion (K. Suzukake et al 1982). Glutathione may also be important with other alkylating agents (H.L. Gurtoo et al 1981). The low molecular weight cytoplasmic metallothionein proteins which are rich in sulphydryl groups can also contribute to resistance and have been implicated in chlorambucil resistance in one study (L. Endresen et al 1983).

Since the DNA of the cell appears to be the target of many cytotoxic drugs the various types of DNA repair have been an important area of research. For the bifunctional agents resistance to melphalan (P.G. Parsons et al 1981; L.A. Zwelling et al 1981), busulphan (P. Bedford et al 1982), cis-platinum (L.A. Zwelling et al 1981) and the nitrosourea CCNU (L.C. Erickson et al 1978) has been reported with reduced DNA crosslinking although different sorts of DNA lesions may be important in each case. Differences in the initial formation of crosslinks seemed

to be important as well as the subsequent temporal repair of them. These results have been criticised by others who found that, while their sensitive cell lines showed increased cross-linking at a particular dose of cis-platinum, at an equitoxic dose the resistant lines showed more cross-links apparently simply indicative of the higher drug concentrations present (M.C. Strandberg et al 1982) implying that either the critical DNA lesion was not measured or there are other causes for the resistance as well. Particular repair pathways will be important for particular drugs, as has been shown with base excision monoadduct repair for nitrosoureas where the so-called Mer phenotype was important to resistance (L.C. Erickson et al 1980). The nuclear matrix itself within the cell may also be important (K.D. Tew et al 1983). It seems likely that in many cases, particularly at low levels of resistance, more than one mechanism of resistance will be acting in concert (A. Begleiter et al 1983) to give the end result of survival against cytotoxic insult and more studies are needed where the different possibilities are investigated together.

1.4 Cisplatinum studies

Cisplatinum has rapidly become an important anti-cancer drug since its introduction into clinical trials in the early 1970s with activity against a number of human cancers (A.W. Prestayko et al (eds) 1980). Its biological

activity was first noticed by Rosenberg and his colleagues in 1965 when they showed inhibition of cell division and abnormal filamentous growth in E. coli by electrolysis products from a platinum electrode (B. Rosenberg et al 1965). When the compound was identified as cisplatinum, (formerly known and synthesised in the 19th century as Peyrone's chloride), its antitumour activity was soon shown in murine sarcoma 180 and L1210 leukaemia (B. Rosenberg et al 1969) and phase I trials began in humans in 1971. Its major toxicities include nephrotoxicity now largely controlled by hydration and mannitol diuresis (I.H. Krakoff 1979), gastrointestinal toxicity, some myelosuppression and neurotoxicity (D.D. Von Hoff et al 1979).

The chemistry of cisplatinum has been recently reviewed (S.J. Lippard 1982). It can undergo a hydrolysis reaction with displacement of the chloride groups in a stepwise manner with water molecules. These aquated complexes appear to be the active species and can react with nucleophiles such as DNA and proteins (N.P. Johnson et al 1980). Pharmacokinetic studies which have mainly only measured the platinum, by atomic absorption spectroscopy, have had to take account of reactions in the plasma especially the binding to protein and so figures for total platinum and ultrafilterable (i.e. not protein bound) platinum are usually quoted (J.J. Gullo et al 1980). Both

total and ultrafilterable platinum have a rapid initial clearance with half-lives less than a half-hour but total platinum has a much slower secondary phase of clearance (half-life 2-5 days). The initial rapid loss of platinum is mainly by excretion in the urine with 60-70% of the total platinum recovered there during the first 4 hours (C.L. Litterst et al 1976). Cisplatinum appears to bind to most plasma proteins (J.J. Gullo et al 1980) but also react with other low molecular weight plasma species (A.J. Repta et al 1980). However 60 to 80% of the ultrafilterable platinum is parent drug (K.J. Himmelstein et al 1981) and work to identify the other metabolites is only in preliminary stages (P.T. Daley-Yates et al 1983). Generally it would appear that the chloride concentration in the plasma (100mM) is high enough to leave the equilibrium of the aquation reaction far to the left but when the drug enters the cell and meets a much reduced chloride ion concentration (5mm) the equilibrium is pushed to the right and the more active aquated complexes are formed and can then react intracellularly (A.F. Le Roy et al 1979) as shown in Figure 1. However others have argued that reactions not dependent on initial aquation can occur in the plasma (A.J. Repta et al, 1980).

There is general agreement that the major target of cisplatinum for its toxicity and anti-tumour-activity is DNA (J.J. Roberts et al 1979). DNA synthesis is inhibited

Figure 1. Aquation of Cis-Platinum

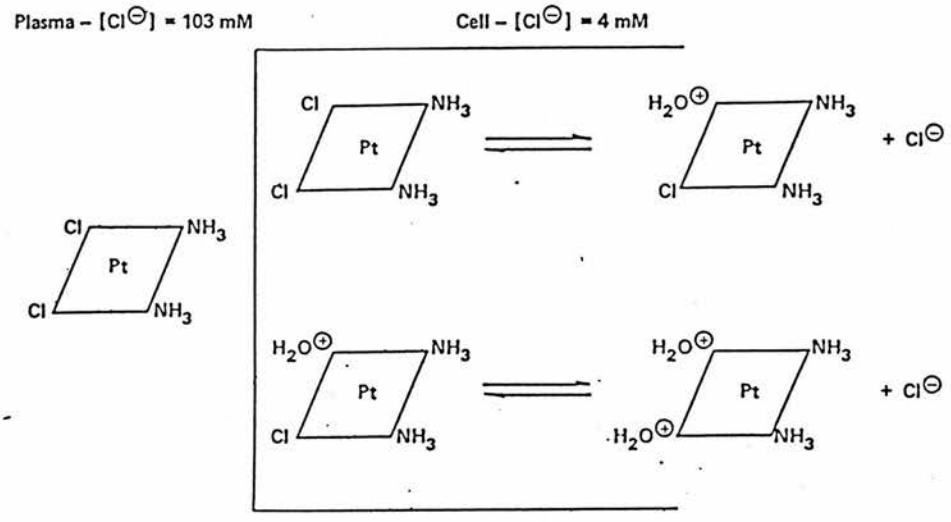
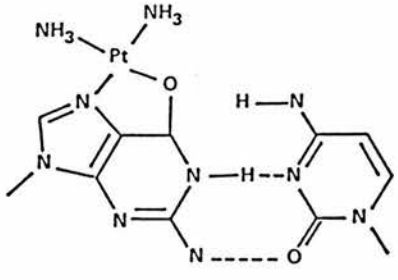
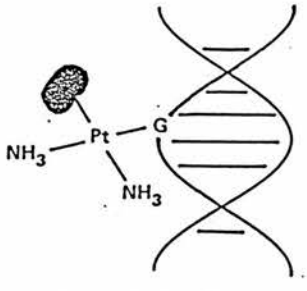


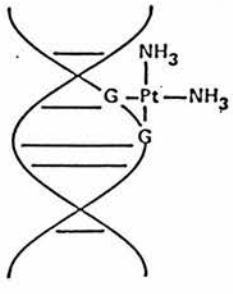
Figure 2. Possible types of DNA interactions with Cis-Platinum



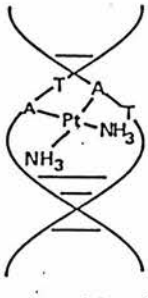
Bifunctional Binding to 1 DNA Base



DNA-Protein Crosslink



Intrastrand Crosslink



Interstrand Crosslink

before and at lower drug concentrations than other cell activities like RNA and protein synthesis (H.C. Harder et al 1970). Subcellular fractionation studies have shown amounts of platinum species are greatest in the cytoplasm (R.P. Sharma et al 1983) but on a per gram of protein basis considerable amounts concentrate in the nucleus (D.D. Choie et al 1980). Using electron microscopy to see the platinum one study has shown platinum concentration in the nucleus (M.U.A. Khan et al 1978). At LD_{37} toxic doses to HeLa cells more platinum is bound to DNA than RNA or protein on a mole per mole basis (although similar amounts on a mole per gram basis, suggesting the DNA is the most sensitive cell target). Only one protein in every 1500 would have undergone reaction at this dose, theoretically the dose just required to kill one cell (J.J. Roberts et al 1979). The precise DNA lesions which are critical to toxicity are still uncertain but bifunctional attack seems to be important. Suggested lesions are shown in Figure 2.

DNA interstrand crosslinks have been correlated with both toxicity and resistance (L.A. Zwelling et al 1981). Others have criticised this work and suggested that, while the extent of interstrand crosslinking was related to the concentration of drug applied, a more cytotoxic lesion must exist (M.C. Strandberg et al 1982) with intrastrand crosslinks being the most likely possibility (S.J. Lippard 1982). The presence of interstrand crosslinks can be

studied by the technique of alkaline elution but the chemical nature of the reaction products of cisplatinum with DNA are only now being elucidated with the N^7 of guanine proving the most reactive centre (J.P. Macquet et al 1983). Intrastrand crosslinks between neighbouring guanines in DNA in vitro have been identified (A.M.J. Fichtinger-Schepman et al 1982). Antibodies to cisplatinum-DNA adducts have recently been prepared and they may provide a way of studying adducts such as intrastrand crosslinks more closely (S.J. Lippard et al 1983). Others have used the radiolabelled analogue cis-dichloro(ethylenediamine)platinum (II) to start looking at these adducts (A. Eastman 1983) and shown the intrastrand crosslink to be quantitatively the most important lesion with calf thymus DNA. By contrast interstrand crosslinks only represent about 1% of the platinum DNA reactions (J.J. Roberts et al 1982) but may be a critical lesion.

Over 1000 cisplatinum analogues have been synthesised and studied and the structure-activity relationships obtained (K.R. Harrap 1983) have suggested the likely cytotoxic lesions briefly discussed above. Other lesions such as DNA mono-adducts or DNA-protein crosslinks are thought to be less important since they can be formed by much less toxic analogues such as trans-platinum with similar efficiency to cisplatinum (L.A. Zwelling et al 1979a). As discussed by Harrap, studies on structure-activity

relationships have revealed that for antitumour activity the complexes should be neutral and have the leaving groups necessary for bifunctional reactivity in the cis rather than the trans orientation of the square planar complex. The two leaving groups such as the chlorides in cisplatinum have an effect on the reactivity of the molecule, while the two inert ligands usually amine derivatives seem to have more effect on the anti tumour selectivity and systemic toxicity (J-P. Macquet et al, 1983a). Various analogues have been designed with increased activity or solubility or decreased nephrotoxicity etc. in mind. Certain platinum (IV) complexes are also active. The chemistry of this platinum group of antitumour agents can be seen to be similar to the classical bifunctional alkylating agents with whom they are often broadly categorised (F.M. Schabel et al 1978) but the reactivity and selectivity show important differences. For example cisplatinum induces many less DNA interstrand crosslinks than sulphur mustards per DNA reaction (J.J. Roberts et al 1979).

Cisplatinum is as toxic (if not more so) to stationary phase as to proliferating cells in tissue culture and longer exposures are more toxic than short exposures of the same dose (J-P. Bergerat et al 1979), suggesting it is toxic at all phases of the cell cycle. However, toxicity is greater towards G_1 phase cells and DNA repair

processes have been invoked to explain this (J.J. Roberts et al 1980). For example interstrand crosslinks appear to be generated in a two step process with the second step taking several hours (L.A. Zwelling et al 1979b) so that their formation will be competing with repair processes even on the mono-adducts where quenching of otherwise reactive adducts could reduce toxicity (K. Micetich et al 1983). DNA repair pathways are best understood in bacteria and various E. coli repair deficient mutants have been shown to be more sensitive to cisplatinum (R. Alazard et al 1982). This sensitivity is due in part to deficient excision repair but other pathways such as postreplication repair would seem to be important as well (J.J. Roberts et al 1979). Increased sensitivity has also been shown in a human excision-repair deficient xeroderma pigmentation cell line (H.N.A. Fraval et al 1978). Cisplatinum is also mutagenic to chinese hamster cells presumably due to DNA lesions and misfunction of repair processes (L.A. Zwelling et al 1979c).

Resistance of cells to the effects of cisplatinum can be attributed to the DNA repair process discussed above. Mono-adduct quenching has been suggested as a possibility (K. Micetich et al 1983). However, other cellular processes could also inhibit the DNA lesions being formed. Transport processes into the cell have not been directly implicated as cisplatinum as a neutral complex, is thought

to pass through the cell membrane by passive diffusion. However, resistance to melphalan in an L1210 sub-line cross-resistant to cisplatinum has been associated with changes in transport mechanisms (W.R. Redwood et al 1980). It is perhaps important to distinguish between cisplatinum resistance associated with cross-resistance to the classical bifunctional alkylating agents such as melphalan and resistance not showing this association (A. Eastman et al 1981). For example Schabel and his colleagues using both L1210 and P388 cells and resistant sublines have shown that sublines selected for resistance against malphalan showed cross-resistance to cisplatinum but sublines selected for resistance against cisplatinum did not show cross-resistance to melphalan (F.M. Schabel et al 1983). Thus the selecting agent is probably important for the mechanism of resistance observed. Levels of SH-compounds, particularly glutathione, have been implicated in resistance to melphalan with deactivation of the reactive agent (K. Suzukake et al 1983) and appear to play some role in the metabolism of cisplatinum. Diethyldithiocarbamate, a sulphydryl group reagent, can inhibit cisplatinum induced nephrotoxicity (R.F. Borch et al 1979). Alterations in renal levels of glutathione and glutathione dependent enzymes can be observed after cisplatinum treatment (C.L. Litterst et al 1982) although this may not be due to a direct interaction of cisplatinum with SH-compounds (J. Levi et al 1980). In rat liver and

kidney appprcximately 50% of the platinum in the cytoplasm of the cell was in the form of low molecular weight species (<1000MW) with about 25% bound to high molecular weight proteins (>20,000 MW) and 25% bound to small proteins of 6000-12000 molecular weight thought to be metallothionein-like proteins (R.P. Sharma et al 1983). Indeed in 2 cell lines selected for resistance against cadmium, cross resistance was observed against cisplatinum and resistance was correlated with an increase in the cysteinyl-rich metallothionein proteins which bind cadmium and platinum (A. Bakka et al 1981). These cells are also resistant to chlorambucil (L. Endressen et al 1983).

Clearly mechanisms of resistance can occur at several levels depending on the selecting agent, the cell system and the random chances of mutations, and variable cross-resistance has been observed even within this class of square-planar platinum complexes (J.H. Burchenal et al 1978). These variations will also have an effect on interactions in combined drug or radiation therapy and various studies have looked for modification of cisplatinum effects with other agents, for example with ionizing radiation (E.B. Douple et al 1980).

1.5 Rationale for this project

The background to this project reviewed above indicates the clinical importance of ovarian cancer. Although responses to chemotherapy have improved considerably, a high proportion of patients relapse and subsequent treatment is unsuccessful. Thus while epithelial ovarian carcinoma is a chemosensitive tumour the development of drug resistance is a major problem leading to shortlived survival and current therapy needs to be further improved. Ovarian cancer cell lines can be grown in vitro from clinical samples and so in this project I decided to use clinical samples and derived cell lines as a model for studying drug resistance. Emphasis has been placed on resistance to cisplatinum since it is thought to be the key component of the best current combination chemotherapy.

2. Characterization of ovarian adenocarcinoma cell lines

2.1 Material already available in the Department

When I arrived in the Medical Oncology Unit in January 1984 some progress had already been made towards a model for drug resistance in ovarian cancer. Ten ascites samples from 7 patients had been collected and 3 cell lines established. Eight of the ascites were from patients with proven ovarian carcinoma, including 3 ascites from 1 patient with a poorly differentiated adenocarcinoma. Cell lines designated PE/01 and PE/04 (PE/0 indicating peritoneal effusion from an ovarian carcinoma and numbered as collected) had been established from the first two ascites from this patient collected in February and December 1982 respectively. Two ascites samples collected 6 months apart were from one patient with a metastatic adenocarcinoma thought to be ovarian. A cell line designated PE/A1 had been established from the first ascites. Samples from all the ascites were available frozen in liquid nitrogen.

Most work had concentrated on characterisation of the 2 cell lines PE/01 and PE/04. The details of the patient´s history and treatment and the times when the ascites were taken are shown in Figure 3. Although the PE/01 ascites was taken upon relapse after a long remission subsequent to initial treatment of the patient,

Figure 3

PATIENT WITH ADVANCED OVARIAN CANCER

CASE HISTORY

Advanced disease with ascites (April 1980)

↓ Surgery

Treatment - Cisplatinum, chlorambucil, 5-FU[a]

Response

(Disease free at 2nd look laparotomy Jan 1981)

↓ Relapse with ascites (Feb 1982) -> cell line PE01

↓ Treatment - Cisplatinum, Chlorambucil, 5-FU[b]

Response

↓

Relapse (Nov 1982)

↓ Treatment - High dose Cisplatinum[c]

No response Further ascites (Dec 1982) -> cell line PE04

↓ Further ascites (Feb 1983) -> cell line PE06

↓

Death (Feb 1983)

(a) 7 courses May-Dec 1980
Cisplatinum 30mg/m^2 and 5-fluorouracil 500mg/m^2, I.V. days 1 and 8.
Chlorambucil 0.1mg/kg x 14 days (but often reduced due to low white blood cell count).

(b) 6 courses March-Aug 1982
Same schedule as in a)

c) Nov 1982
Cisplatinum 100mg/m^2 I.V. then 50mg/m^2 4 weeks later.

d) Jan 1983
1gm 5-fluorouracil I.P.

a further response was apparent until the relapse when the PE/04 ascites was obtained. The ascites and the derived cell lines thus seemed a good initial model for studying drug resistance in human ovarian carcinoma cells.

Cytological characterisation of these 2 ascites, together with the original surgical specimen from the patient's operation in April 1980, had been carried out by Dr. Margaret McIntyre, Consultant Pathologist in the Department of Pathology, Western General Hospital. The cytology of the initial cultures of PE/01 before a cell-line was established and of very early passages of the PE/04 cell line had also been analysed with Papanicolaou staining. Cultures of cuboidal type cells, with fibroblastic cells in the initial cultures, were observed with some vacuolated cells and some "signet-ring" cells characteristic of ovarian ascites. Mucin production was noticed in a few cells by staining by the periodic acid-Schiff reaction (P.A.S.) with diastase.

Initial karyotyping was done by Dr. Karin Buckton of the MRC Clinical and Population Cytogenetics Unit. Both cell lines were hypodiploid with a modal chromosome number of 41 but a considerable range. Abnormal chromosomes in common were 3,9,13 and 22 but PE/04 had a chromosome 8 and 17 not identifiable in PE/01. Chromosome spreads were prepared at passages 5,9,22 and 42 for PE/01 and 7,15,17

and 23 for PE/04 with similar results on different passages.

A clonogenic assay using the Courtenay procedure (V.D. Courtenay and J. Mills 1978) had been established and preliminary experiments on sensitivity to cisplatinum, chlorambucil and 5-fluorouracil done since these were the drugs with which the patient had been treated. These experiments suggested a difference between the cell lines in sensitivity to cisplatinum although problems had been experienced especially in testing PE/01 due to its low and apparently variable plating efficiency. Attempts to establish a xenograft from the PE/01 cell line had failed although on two occasions some growth was observed but the nodules then regressed.

2.2 Characterisation of cell lines PE/01 and PE/04

Some characterisation on these cell lines, as indicated above had already been done. It was necessary to complete the cytology and cytogenetics and improve the cell growth in agar so that reliable results could be obtained in the Courtenay assay. Experiments to assess the growth of the cell lines under a variety of conditions in vitro were also required and other cellular features including cell volume, DNA content, antigenic determinants, hormone receptors and Mer phenotype were also characterised.

2.2.1. Cytology

Light microscopy

The cell line designated PE/01 took approximately 6 months to become established, possibly due to its sensitivity to trypsin and several samples were analysed during this period by various stains including May-Grunwald Giemsa, Toluidine Blue, and the special stains Sudan black for lipid, Southgate mucicarmine for mucins, and Alcian blue for acid mucopolysaccharides. However, most preparations were stained using the Papanicolaou technique for morphological detail or by the periodic acid - Schiff (P.A.S.) reaction for mucopolysaccharides and mucins with and without diastase which removes glycogen. These initial cultures showed characteristic epithelial cuboidal and some multinucleate cells together with more elongated fibroblasts which gradually died out. Some "signet-ring" cells were observed (with the nucleus pushed into a crescent on one side by a large vacuole) as well as other vacuolated cells. Some but not all the cuboidal and vacuolated cells were positive with P.A.S. - diastase indicating mucin in the cytoplasm. In the cuboidal cells the large oval nuclei were normally central with prominent and irregular nucleoli and often multiple nucleoli. No changes were observed with passaging of the cells and cells at passage 78 showed a similar profile. The number of vacuolated cells seemed to vary with the culture conditions with more observed in confluent cultures.

Ascites preparations showed more "signet-ring" and vacuolated cells than the cultured cells. Their cytology was unaffected after freezing in liquid nitrogen.

The cells in cultures of PE/04 showed similar cytological features to PE/01. Cuboidal and vacuolated cells at passages 2-4 showed the features noted above. PE/04 grew out in culture much more rapidly than PE/01 with cytological analysis in both cell lines being done by Dr. McIntyre approximately 3 months after cultures were initiated. No differences were observed between parallel cultures of PE/04 which had arisen from different wells and been kept separate. Part of the initial PE/04 cultures also grew in suspension in clusters of closely packed cells. Cytology of suspension cultures at passage 1 showed similar features to those above although in this sample the P.A.S. positivity was removed by diastase. The monolayer cultures at later passage (passage 48) showed similar features to the early passage cells and again no changes were observed with passaging.

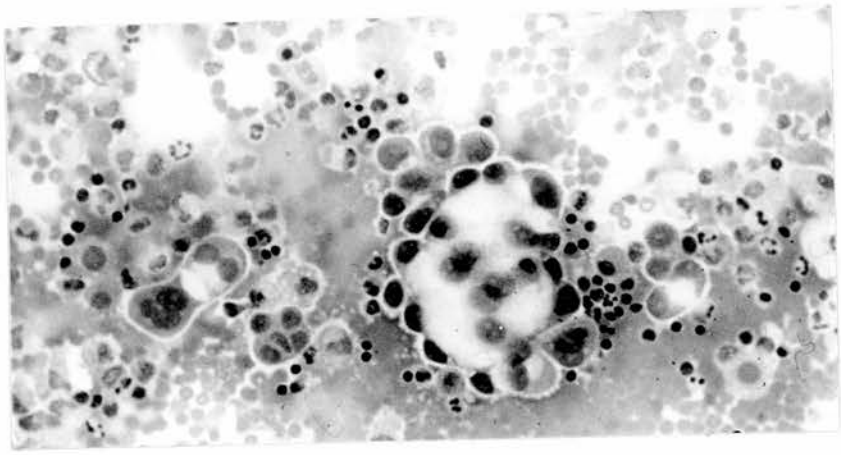
Some typical examples of the cytology of the ascites and cell lines are shown in Figures 4 and 5.

Electron microscopy

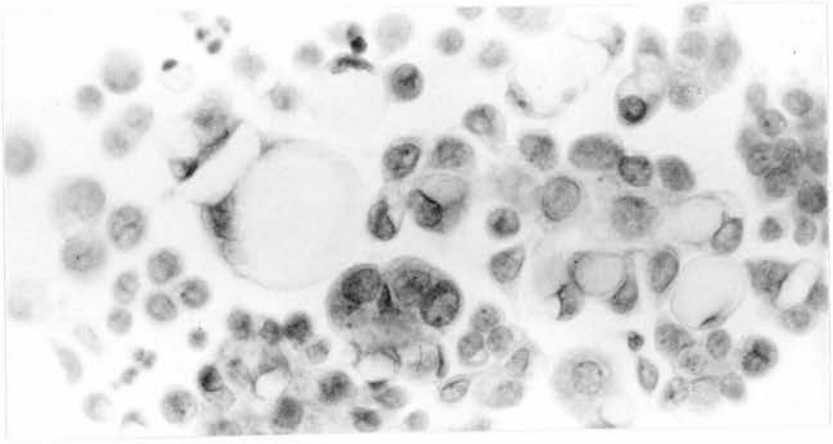
Both cell lines were also observed at the ultrastructural level. Initial experiments involved harvesting cultures

Figure 4 Cytology of Ascites

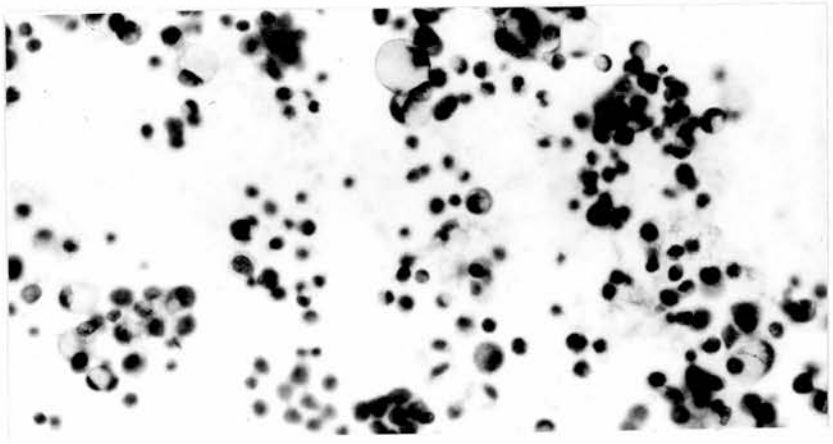
(Papanicolaou stained)



4a. Original ascites, April 1980 (mag. x900)



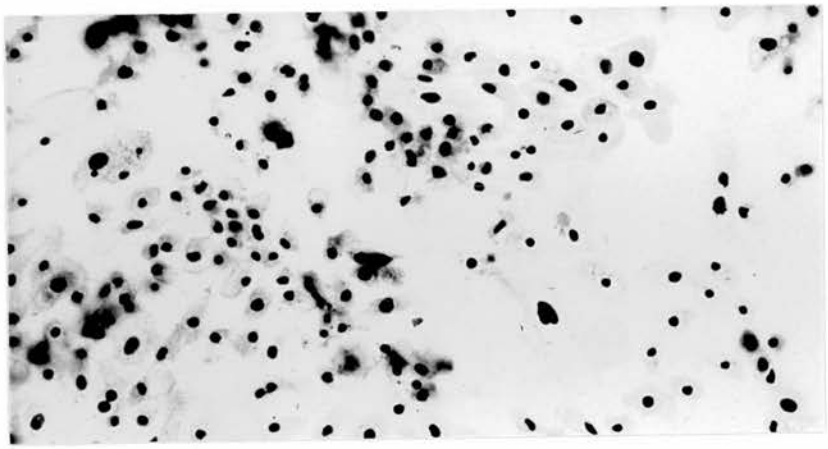
4b. PE/01 ascites, Feb 1982 (mag. x1400)



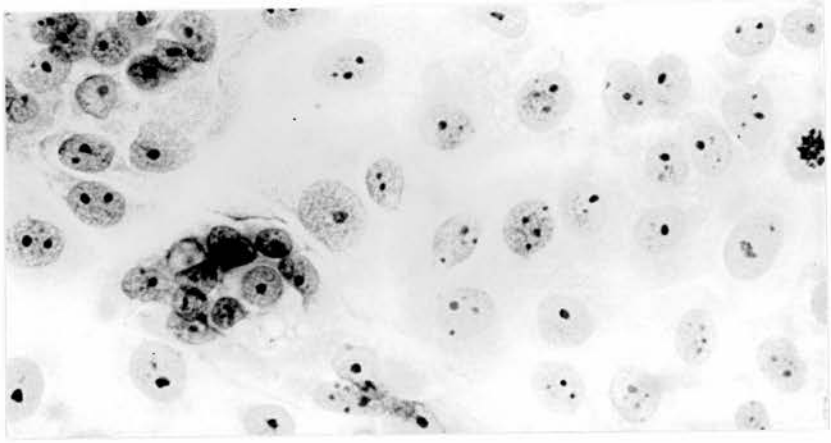
4c. PE/04 ascites, Dec 1982 (mag. x900)

Figure 5 Cytology of Cell lines

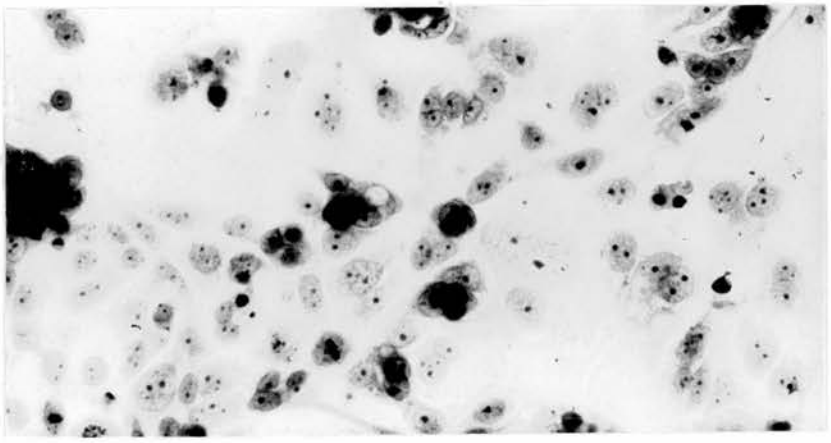
PE/01
passage 1
(mag. x900)



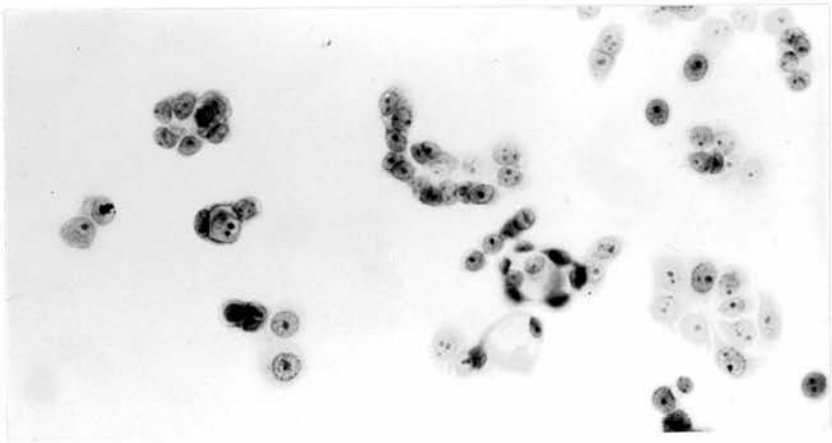
PE/01
passage 79
(mag. x1400)



PE/04
passage 2
(mag. x900)



PE/04
passage 48
(mag. x900)



by trypsinization to a cell pellet. For transmission E.M. cells were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer pH7.3, post fixed in 1% osmium tetroxide, dehydrated through increasing ethanol solutions, embedded in araldite, sectioned on a LKB V ultratome, stained with uranyl acetate plus lead citrate and viewed on a Philips EM300. To look at cell surface features by scanning E.M. cells were fixed and post fixed in the same way, dehydrated through acetone, critical point dried from liquid CO_2 (Balzer Union), gold coated on a sputter coater (Polaron E5001) and viewed on a Cambridge Stereoscan 180. In later experiments cells were grown on plastic coverslips in Leighton Tubes (Costar) and fixed in situ. Processing was similar to above with the coverslip being peeled away from the cells after embedding in araldite (G. Seman et al 1975) to then allow horizontal and vertical sections to be cut through the cell monolayer for transmission E.M.

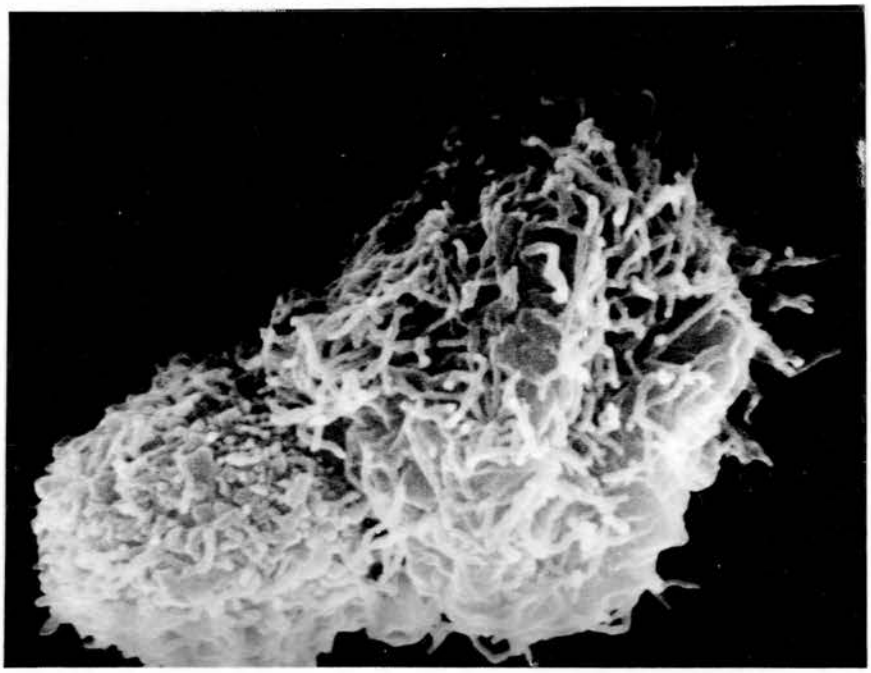
Cells from both lines show large sometimes convoluted nuclei with prominent nucleoli and dark nuclear margination. The cell surface shows many microvilli under scanning E.M. and under transmission E.M. these could also be seen. Sometimes the cells were extremely indented at one end with microvilli concentrated there. Some desmosomes between cells were observed. Some cells had many organelles and vacuoles while others had relatively

featureless cytoplasm. Also mitotic cells could be seen. Many pale rounded inclusions in some cells appeared to be lipid droplets or mucinous secretions. The in situ sections show quite dilated intercellular spaces and some possibly non-cellular inclusions. The monolayers show some overlapping of adjacent cells and occasional multi-layering. These features were similar in both cell lines and consistent with the light microscopy cytology. Examples of these features are shown in the E.M. photographs in Figures 6 to 10.

2.2.2 Cytogenetics

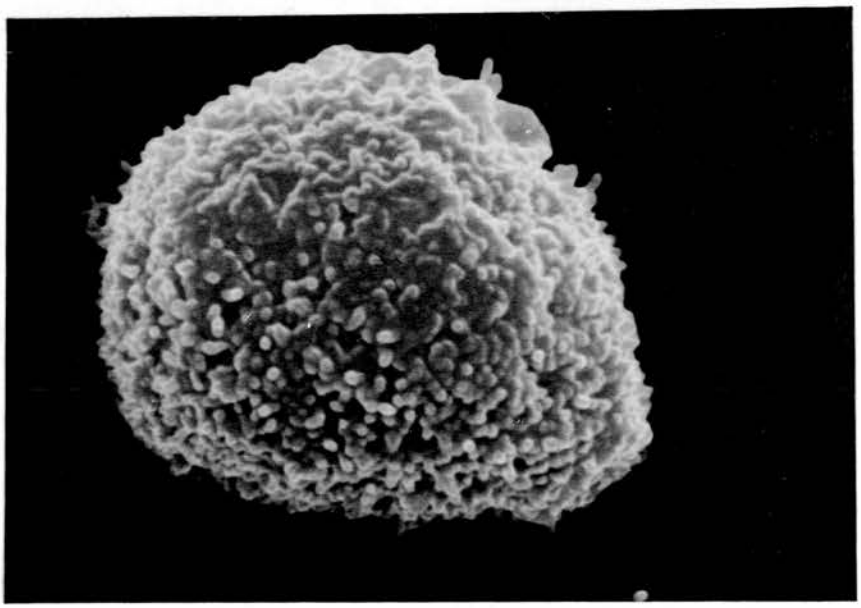
Chromosome preparations were obtained by a routine air-drying procedure by Ms. Sandra Lawrie and analysed for chromosome number per cell. They were stained for Q-banding with spermadine bis-acridine by the technique of Van de Sande (J.H. Van de Sande et al 1979) and analysed by Dr. Karin Buckton of the M.R.C. Clinical and Population Cytogenetics Unit. The PE/01 cell line was analysed at passages 5,9,22 and 42 and PE/04 at passages 7,15,17 and 23. Although the populations were karyotypically heterogenous a number of consistent abnormalities were identified. Both cell lines were hypodiploid with a modal chromosome number of 41. This compared with a normal 46 chromosome karyotype prepared from the patient's peripheral blood leucocytes. This blood sample was taken in January 1983 not long before the patient died and after

Figure 6 Scanning Electron Microscopy



3um scale bar

PE/01 passage 79 (trypsinised cells)



3um scale bar

PE/04 passage 48 (trypsinised cells)

Figure 7 Transmission Electron Microscopy
PE/01 passage 79, trypsinised cells
(mag. x5100 approx)

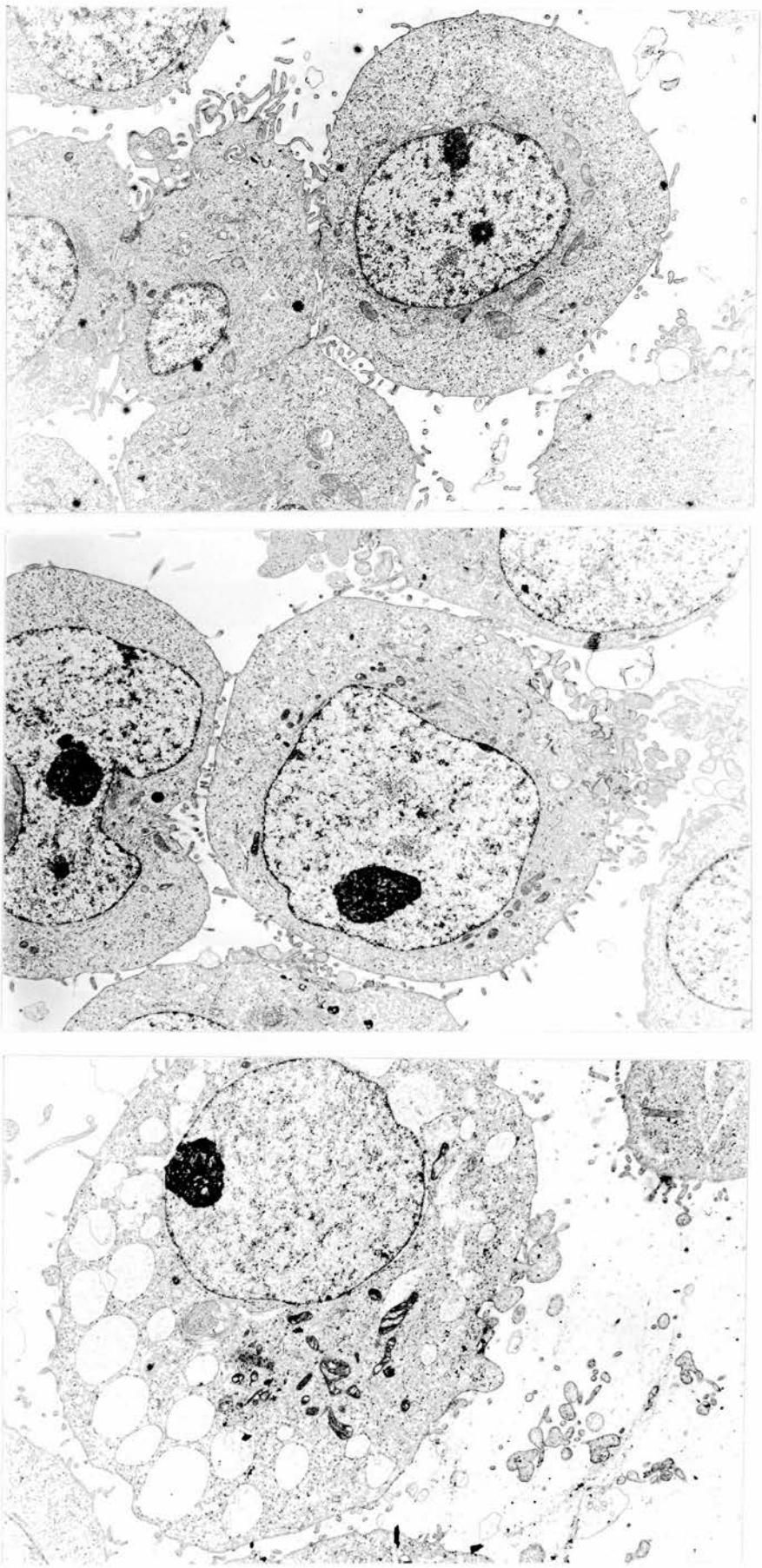


Figure 8 Transmission Electron Microscopy
PE/04 passage 48, trypsinised cells
(mag. x7600 approx)

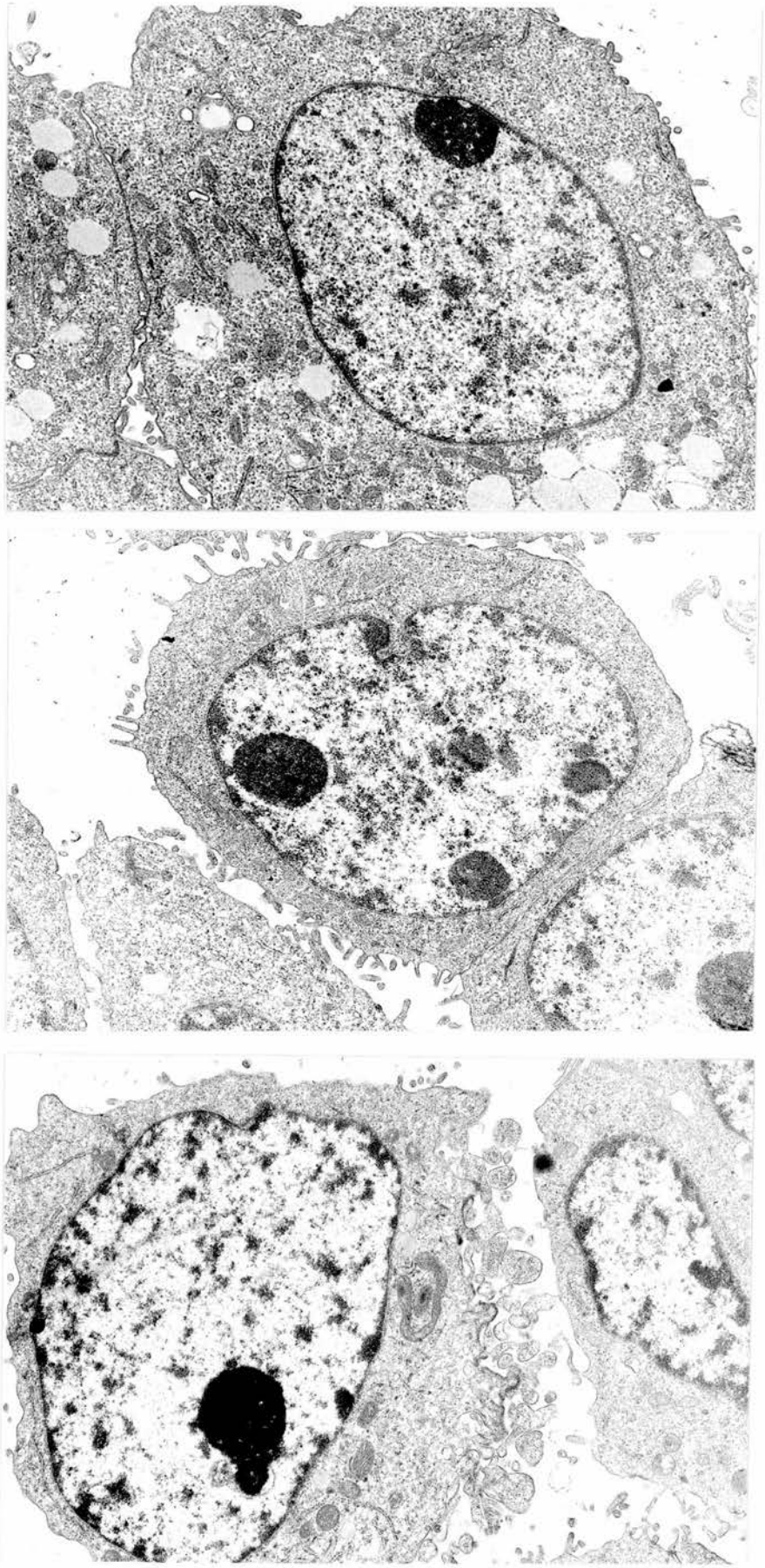
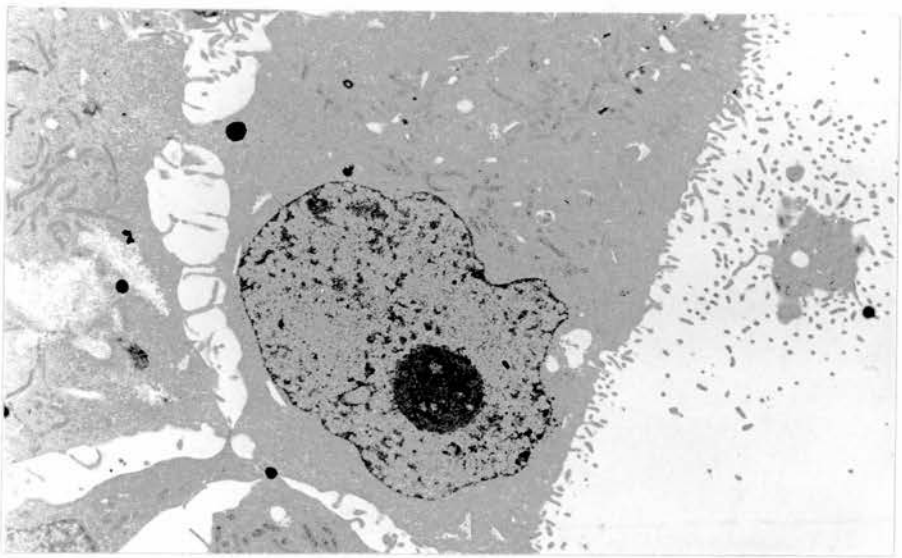
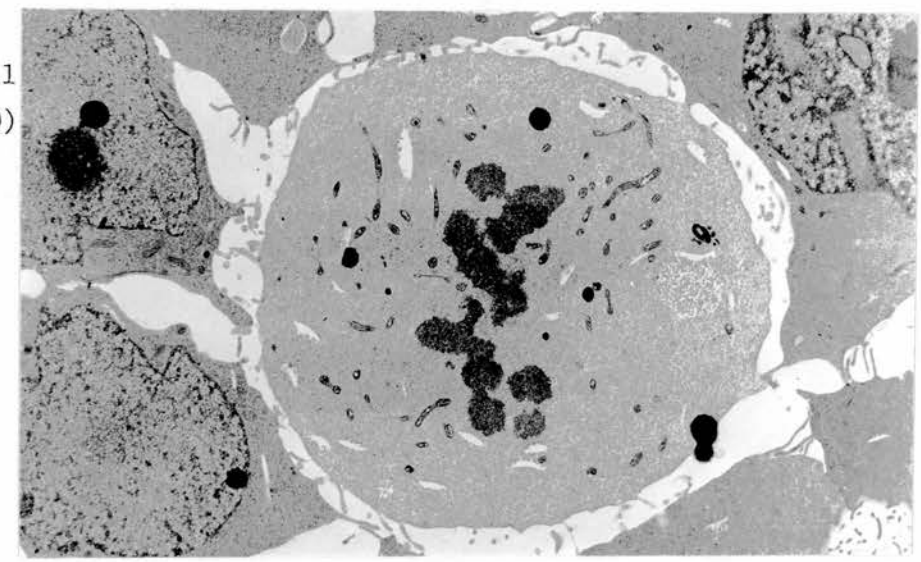


Figure 9 Transmission Electron Microscopy
PE/01 passage 79, in situ

Horizontal
Section
(mag. x4200)



Mitotic cell
(mag. x4200)



Vertical
Section
(mag. x4200)

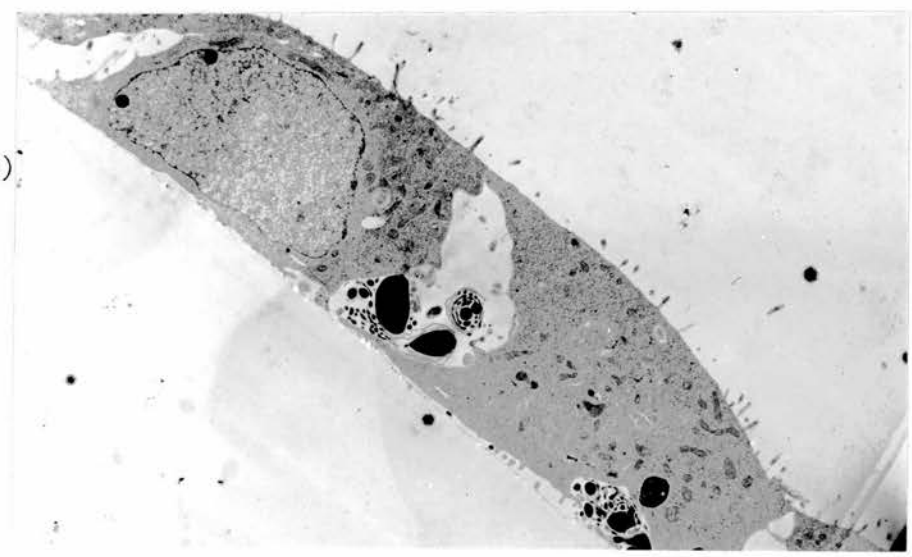
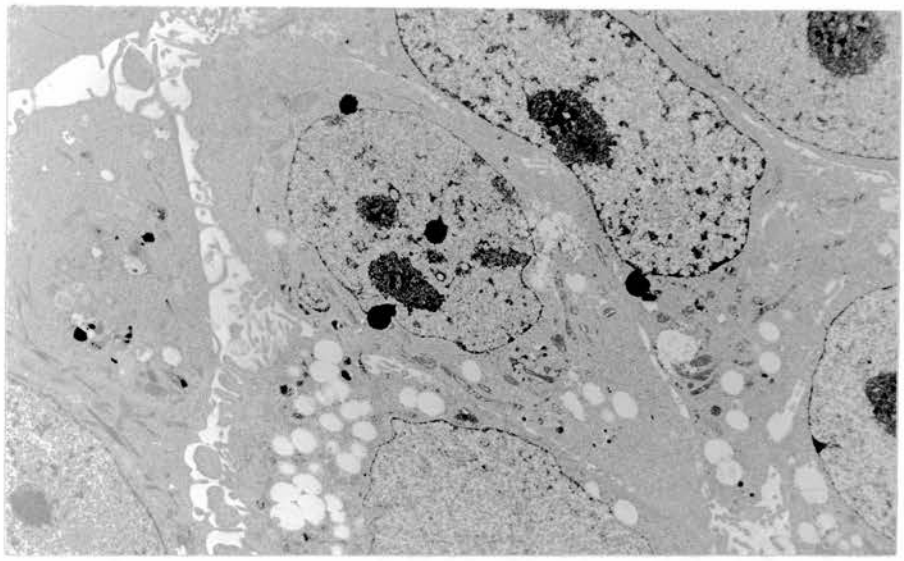
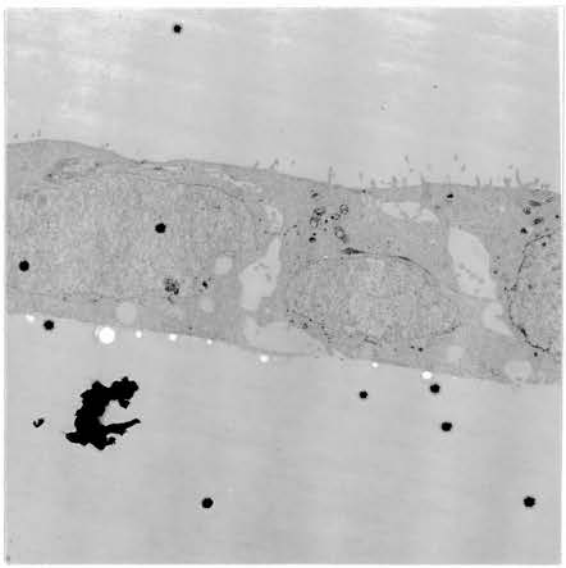


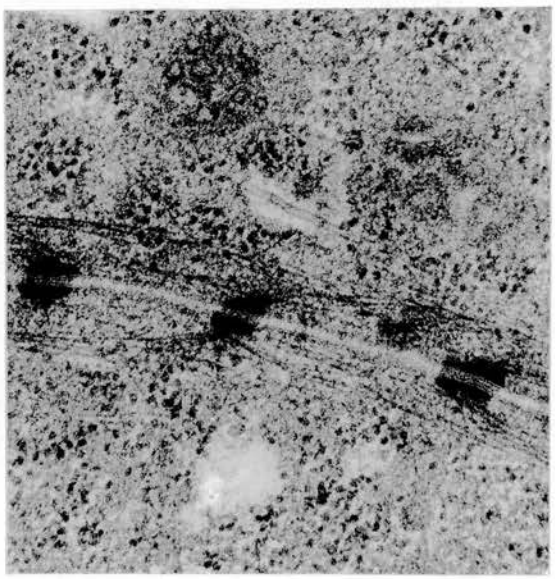
Figure 10 Transmission Electron Microscopy
PE/04 passage 48, in situ

Horizontal
Section
(mag. x3500)



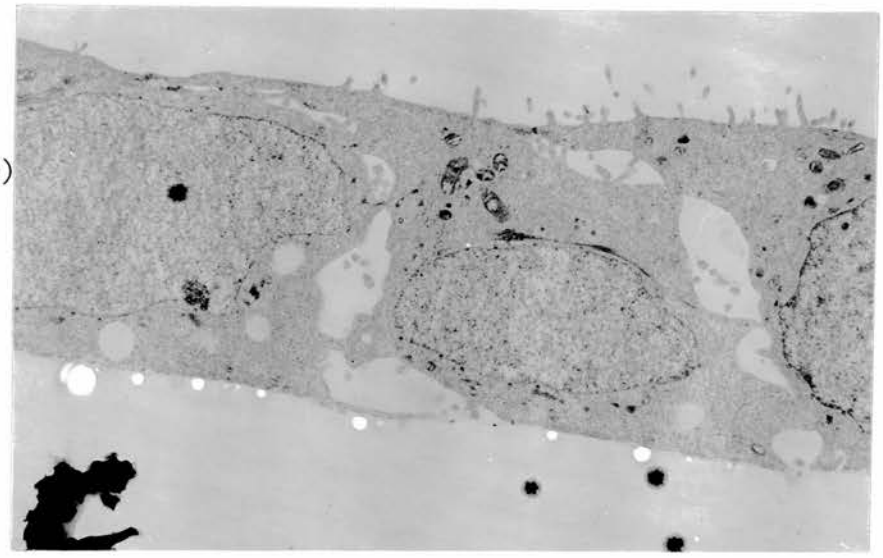


Vertical Section
(mag. x3800)



Horizontal Section showing
membrane desmosomes (x52000)

Vertical
Section
(mag. x6900)



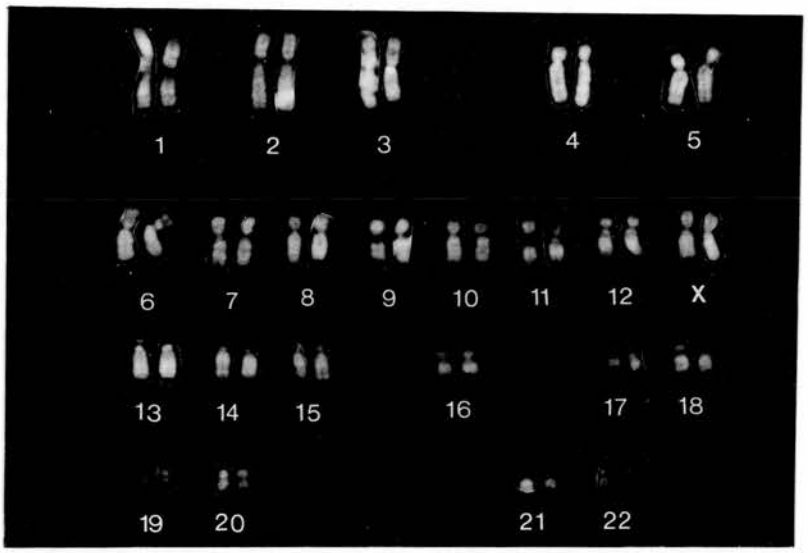
the PE/04 ascites had been tapped.

It is perhaps best to quote the results from Dr. Buckton's analysis and her conclusions on the significance:

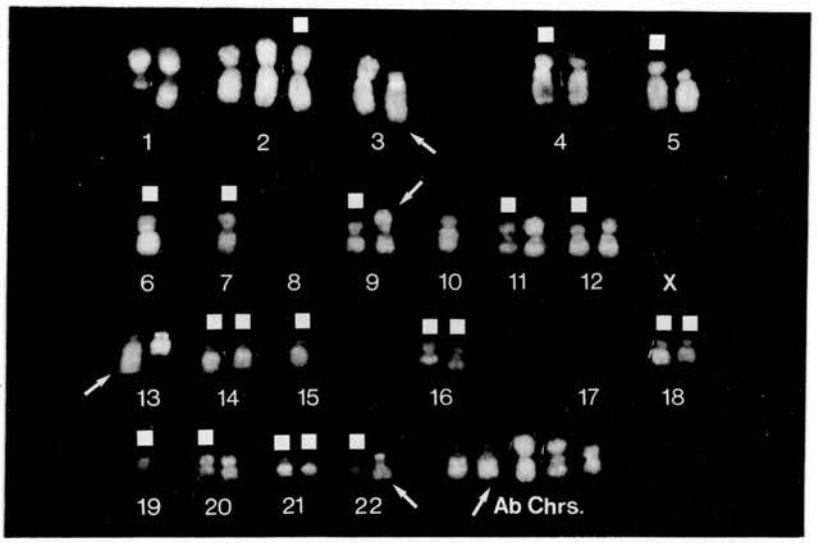
> "Chromosome preparations stained for Q-banding with spermadine bis-acridine by the technique of Van de Sande et al (1979) were examined from the peripheral blood leucocytes and cultures grown from ascites material taken from a patient with ovarian carcinoma before and after she acquired drug resistance. The cells from the peripheral blood showed a normal 46,XX karyotype, whereas the ascites cultured cells were hypodiploid, the chromosome numbers varied between 39 to 42. Examples of the chromosome complement before resistance (PE/01) and after (PE/04) with 41 chromosomes are illustrated (Figure 11b,c). Figure 11a is a fluorescent karyotype made from a normal peripheral blood cell. The cultured ascites cells in addition to being hypodiploid, contained many abnormal chromosomes. In Figures 11b and 11c the chromosomes that are considered to be normal are marked with a white square. PE/01 has only 20 such chromosomes; none of the cells from this culture contained a normal #8 or #17 chromosome nor any material that could be considered as coming from either of these chromosomes, although this cannot be

Figure 11 PE/01 and PE/04 Karyotype

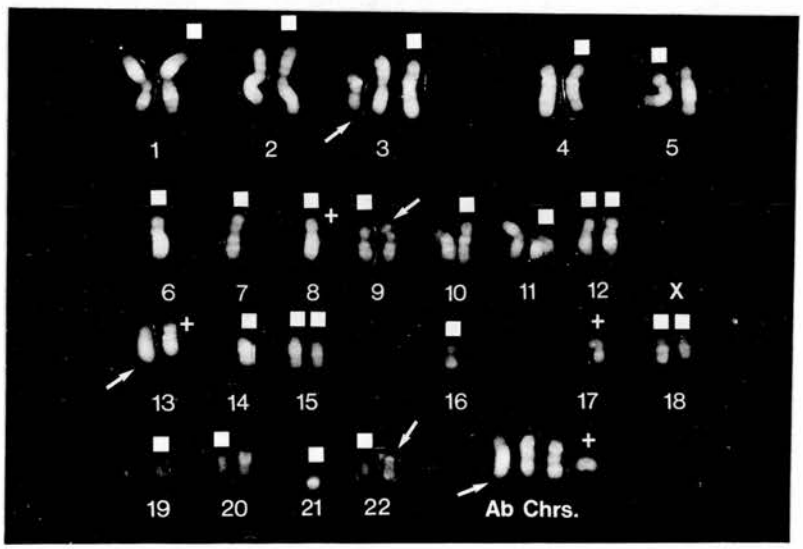
11a.
Peripheral
lymphocyte



11b.
PE/01



11c.
PE/04



excluded because there are so many rearranged chromosomes. PE/01 had 12 consistent abnormalities which were:- both #3's inv(3)(p-q+),5p-,9p+,11p+, both #13's 13q- and 13q+, 22q+ and 4 chromosomes of unknown origin. The number and appearance of the other abnormal chromosomes varied. The polymorphic Q-bands on chromosomes #3 and the acrocentics allowed us to distinguish the origin of some of the abnormal chromosomes. From Figure 11a it can be seen that in the normal cells the metacentric #3 had different polymorphic Q-bands, one was intense and the other brilliant (Paris Conference 1971). Both #3 in the tumour were non metacentric, the one with the brilliant Q-band was almost acrocentric, both rearrangements could have been pericentric inversions (inv(3)(p-q+) but with the breakpoints at different positions. The acrocentric pair #13 were also very distinct; one had a brilliant short arm (p11), almost no stalk (p12), and a pale satellite (p13), it is this chromosome that appears to have an interstitial deletion of the long arm (13q-) in the tumour. The other normal 13 has a brilliant p11, long p12 and intense p13. These features were not seen in the tumour. The other chromosome that was thought to be #13 material was a long acrocentric that had pale short arms with few features. This chromosome may have been misclassified, but will be called 13q+.

Both the #22 have intense satellites in the normal cells; only one of these is seen in the tumour and it has additional material on the long arm (22q+). There were 4 other consistent abnormalities: 2 marker chromosomes with very similar appearance - two prominent bands on the distal long arm, but the pericentric chromatin was very pale, uniformly staining, reminiscent of homogeneous staining regions (HSR), these regions were not C-band positive. Also two chromosomes that appeared to be isochromosomes being metacentric with a very similar banding pattern each side of the centromere; one was half the size of the other.

In contrast, PE/04 had an apparently normal member of each chromosome pair including a chromosome #8 and #17, but excepting an X and #13 chromosome. However, the 13q- chromosome now has material translocated to the brilliant p11, which could make this chromosome complete although rearranged. Of the consistent abnormalities seen in PE/01, the following are seen in PE/04:- inv(3)(p-q+) with the brilliant Q-band, 5p-, 9p+, 11p+, 13q+, 22q+, one of the marker chromosomes with an HSR and in many cells the smaller of the two isochromosomes was seen. PE/04 had acquired a ring chromosome, which was approximately the size of a D group chromosome, also other

rearranged chromosomes.

The "normal" #22 in both PE/01 and PE/04 has lost the intense satellites. Deletion of satellites or the whole of an acrocentric short arm is often found as a constitutional "abnormality" in normal individuals, therefore this chromosome material is considered non essential for normal development. Short arm material from other of the acrocentrics in the tumour cells may have been deleted also. In PE/04, neither the #14 nor #15 seem to have the same short arm features as those seen in the normal cells. Despite the loss of the short arm material the #22, #14 and #15 are considered normal.

The chromosome abnormalities found in the tumour cells of other patients with ovarian carcinoma have also been bizarre and inevitably some of the rearranged chromosomes in these tumours have involved the same chromosomes as in the tumour cells from the present case (S. Kakati et al 1975, R.S. Freedman et al 1978, M.F. Van der Riet-Fox et al 1979, L.K. Woods et al 1979, J.M. Trent et al 1981, T.C. Hamilton et al 1983, S.M. Hill et al 1984). However, we did not find the (6;14) translocation which it is suggested may characterise this carcinoma (Human Gene Mapping 7: 1984).

No double minutes were found in these cells. The appearance of two marker chromosomes in PE/01 was consistent with them carrying HSR. HSR´s are usually associated with the acquisition of drug resistance (for review see P.E. Barker 1982), therefore it is somewhat surprising that the number of HSR´s was reduced in PE/04. PE/04 appears to contain more "normal" chromosomes than the earlier culture of ascites cultured cells. Since both cell lines have several rearranged chromosomes in common and had very heterogeneous cell populations, it could be argued that the two cell lines probably arose from a common ancestor, both diverged and co-existed in the body, but that PE/01 was more susceptable to the drug treatment and was overgrown by PE/04. It is very unlikely that PE/04 evolved from PE/01, as it would be almost impossible to regain a normal #8 and #17 having once lost them."

2.2.3 Growth in vitro

My first priority was to assess how well the cell lines we had would grow under experimental conditions and what manipulations could be done without damaging the cells. When I began work the then current passages of the cell lines PE/01 and PE/04 were passage 63 and 32 respectively. In April 1984 it was decided not to continue passaging these 2 lines indefinitely and a cell bank in liquid

nitrogen at their current passages was built up. Cells were then routinely cultured for 5 passages before a fresh sample was taken from liquid nitrogen. Thus all later experiments were done with passages 77-82 for PE/01 and passages 46-51 for PE/04 unless otherwise stated.

Trypsin sensitivity

PE/01 was thought to be sensitive to trypsin since initial cultures had appeared to be severely retarded after trypsinisation. They grew better if the cells were passaged by being scraped off in versene (0.02% EDTA) with a rubber policeman after a wash in Dulbecco's phosphate buffered saline without calcium and magnesium (PBS). However in my experiments on PE/01 at passage 63, cells grew equally well after scraping in versene or after detachment by half strength or normal strength (0.2%) trypsin in versene. Solutions of collagenase/dispase (0.1 U/ml collagenase, 0.8 U/ml dispase in PBS, Boehringer Corp. Ltd) also proved to be suitable. Viability of detached cells was 98-100% by nigrosin staining. Clearly later passages of PE/01 were not sensitive to trypsin but it is not known precisely when this change occurred, possibly as PE/01 became better adapted to growth in vitro during its period in culture. The PE/04 cell line never showed any sensitivity to trypsin.

Confluent cultures

Confluent cultures were observed at passages 63 and 79 for PE/01 and 32 and 48 for PE/04. Both PE/01 and PE/04 showed contact inhibition at confluence although some multi-layering was discernable so that cultures were more than a monolayer thick in parts. Also cells as well as cell debris were found floating in the medium and this increased under nutritional deprivation. When the medium was not replenished many cells detached from the adherent monolayer of confluent PE/01 cultures into the medium but PE/04 maintained a healthy looking monolayer for much longer before many cells started detaching. Thus PE/04 seemed to be more robust than PE/01 when stressed by poor culture conditions.

Sub-clones

Single cell suspensions of PE/01 and PE/04 at passages 66 and 35 respectively were plated out at low density in agar as described in chaper 3 for the Courtenay assays. Colonies grown up from these single cells could then be plucked from the agar using a sterile drawn out pasteur pipette manipulated by hand under a microscope at low magnification and the single colony transferred to a well in a microtitre or 24 well plate. Cells then grew out from the agar plug and became established growing on the plastic surface with liquid medium. Each well of the plate could be checked under the microscope to see that

only 1 colony had been transferred to it. One clone of each of the 2 cell lines was derived in this way. These cells were then grown up, and passaged into dishes and then flasks until enough cells could be obtained for storing in liquid nitrogen. Unfortunately the PE/01 clone was lost through contamination during this process but a PE/04 clone was obtained and stored for future use. In any case it was apparent that this cloning technique could be successfully applied with these cell lines. Much later in the course of this project 6 clones of PE/01 from passage 78 and 10 clones of PE/04 from passage 47 were derived by Ms. Sandra Lawrie using this technique.

A different "limiting-dilution" technique for cloning cell lines was also attempted. Here cells from both cell lines (at passages 78 and 47 for PE/01 and PE/04 respectively) were diluted to a concentration of 10 cells/ml in a single cell suspension and 0.1ml aliquots plated out in the wells of a microtitre plate. These wells were then checked later in the day to score those wells which actually had 1 cell in them and these wells observed over the succeeding weeks. Only about 30% of wells actually had 1 cell in them while about 50% of wells had no cells. No colonies grew up from 60 wells plated for PE/01. For PE/04 colonies grew in a number of wells but subsequently died. However continued growth in 2 wells and subsequent expansion of these 2 populations led to the derivation of

2 PE/04 clones by this method. One clone grew from a well which had 2 cells in it (one on each side of the well) but cell division and growth into a colony was observed from only one of them. Thus this technique was not very successful with these cell lines and the clones obtained may not be very representative of the population from which they came owing to their severe selection. This technique was not pursued further and agar cloning was the preferred method.

Perhaps it should also be noted here that two PE/01 cell lines were originally derived, one of which was not cultured past passage 5. However both were thought to be clonal or near clonal in origin since they developed from single colonies in a tissue culture well. On the other hand eleven parallel PE/04 adherent cell lines and two suspension culture cell lines were originally derived of which only one adherent cell line has been cultured past passage 9. These lines were thought to be polyclonal due to the number of epithelial cell colonies which grew in the initial tissue culture wells.

Colony forming efficiency - plastic

The plating efficiency of the two cell lines was assessed at different cell concentrations and plating densities in various dishes and media. The usual medium was RPMI 1640 containing 10% (v/v) foetal calf serum with added insulin

(2.5ug/ml) and buffered with up to 12.5mM 3-[N-morpholino]propanesulfonic acid (MOPS). The antibiotics streptomycin (100ug/ml) and penicillin (100 IU/ml) were routinely added. Cells were cultured in a high humidity incubator with a 5% CO_2/air gas phase at 37°C.

In 24 well plates (Nunc, Gibco Ltd) with a 1.9cm^2 plating surface area, colony forming efficiency (CFE) was 0.8% for PE/01 (passage 63) compared with 5.7% for PE/04 (passage 32) when 10^3 cells were plated out in 1ml medium. When only 500 cells were plated no PE/01 colonies formed but CFE was still 4.2% for PE/04. At 5 x 10^3 cells or greater too many colonies to count were observed for both cell lines. The colony forming efficiency for PE/04 was obviously greater than for PE/01 at low plating densities and at least for PE/01 the efficiency improved with the number of cells plated. In 50mm diameter dishes with approximately 20.8cm^2 plating surface area PE/04 again showed a superior CFE at low plating density to PE/01 and here the increase in CFE with increasing cell number plated could be clearly seen (Table 4). In addition the CFE in the 20.8cm^2 dishes was lower than that in the 1.9cm^2 wells on a cells plated/cm^2 basis (10^3 cells/well and 1.1 x 10^4 cells/dish both being approximately 500 cells/cm^2 of plating surface).

Six well plates (Linbro, Flow Laboratories) with 35mm

TABLE 4

COLONY FORMING EFFICIENCY OF PE/01 AND PE/04 IN 50MM DISHES

Cell no. plated (in 3mls)	Colonies formed PE/01 (CFE)	PE/04 (CFE)
10^5	Too many colonies to count	
3.3×10^4	426 est. (1.3)	1632 est. (4.9)
1.1×10^4	12 (0.1)	128 (1.2)
3.7×10^3	2 (0.05)	13 (0.35)

CFE = % colony forming efficiency

(est = estimate. With large numbers of colonies some colony overlap occurs making colony counting more difficult).

wells (surface area 9.6cm^2) were found most suitable for the drug assay reported in Chapter 3. Initial work (PE/01 passage 66 and PE/04 passage 36) showed improved CFE by the addition of sodium pyruvate (1mM) but no significant increase with nicotinamide (200uM) as shown in Table 5. Medium RPMI 1640 contains no pyruvate and only 8uM nicotinamide although some contribution can be expected from the foetal calf serum. Pyruvate is well recognised as a growth factor in vitro especially at low cell densities (K.A.O. Ellem et al 1983) and nicotinamide has also been shown to improve colony forming efficiencies at the concentration used here (P.G. Parsons et al 1983). Thereafter 1mM sodium pyruvate was routinely added to the medium when plating cells at low density. Plating cells at densities to give 100-200 colonies in the 35mm wells was used extensively for drug assays in Chapter 3. Plating cell s in 2mls of medium gave a more even spread of cells across the well (medium swirls around less in carrying plates to the incubator before the cells attach) but using 3mls of medium gave slightly better colony growth (3.4% CFE versus 2.4% CFE on passage 78 PE/01 cells in one experiment). A good compromise was found to be plating cells out in 2mls and then adding another 1ml of medium the next day with medium changes every 2-3 days thereafter. Using this system over the following 2 years in some 50 assays colony forming efficiences were 3.9 $\pm$2.3% for PE/01 (passage 77-82) from approximately 4-6 x

TABLE 5

EFFECT OF ADDITIVES ON COLONY FORMING EFFICIENCY

	Colonies formed	
Additive	PE/01 (CFE)	PE/04 (CFE)
None	0.0	256, 233 (2.4)
Sodium pyruvate (1mM)	51, 40 (0.45)	798, 768 est (7.8)
Nicotinamide (0.2mM)	0,0	271, 439 (3.5)

(10^4 cells plated in 2mls medium/9.6cm^2 well)

10^3 cells plated and 8.1 $\pm$3.8% for PE/04 (passages 46-51) from approximately 2 x 10^3 cells plated. The CFE for PE/01 shown in Table 5 was below our general experience and may simply be attributable to poorer technique in a first experiment. Slightly higher CFE in the central wells of plates was also sometimes noted as well as variations between plates from different suppliers. Later during this project a new incubator was acquired which allowed us to vary the oxygen tension. PE/01 and PE/04 cells grown at low density in 5% O_2, 5% CO_2 and 90% N_2 instead of 5% CO_2 in air both showed a 2-3 fold increase in colony forming efficiency.

Colony forming efficiency-agar

Colony growth was also observed from PE/01 and PE/04 cells suspended in 0.3% soft agar under the conditions of the Courtenay assay (V.D. Courtenay and J. Mills 1978 - Hams F-12 medium with 10% foetal calf serum and August rat red blood cells in 5% O_2, 5% CO_2 and 90% N_2). This assay had been previously set up in this laboratory by Ms. Sandra Lawrie and after my arrival we decided to pursue it to obtain drug sensitivity data comparable to other groups. The colony forming efficiencies are reported here and more detail regarding this assay is shown in Chapter 3. Colony forming efficiency improved with passage number. PE/04 again had higher CFEs than PE/01 and gave more consistent results.

At early passages (passages 1-10) PE/04 gave CFE of 1-5% but PE/01 gave CFE of only 0.01-1%. At late passage this had improved to 3.9 ± 2.5% for PE/01 and 18.3 ± 10.4% for PE/04 (passages 77-82 and 46-51 respectively). These changes are illustrated by the experiment shown in Table 6 when we went back to early passage cells to compare drug sensitivities. Colony forming efficiencies shown are the average of 5 replicates.

Doubling time

5×10^3 cells per well were plated out in 24 well plates (Nunc, Gibco Ltd), let grow for 4 days, then quadruplicate wells harvested and counted using a Coulter counter at the same time each day for the succeeding 9 days during which time exponential growth was observed. The doubling time calculated was 53 hours for PE/01 and 59 hours for PE/04. Less detailed previous experiments on earlier passages had indicated doubling times of 68 hours for PE/04 at passage 33, 44 hours for PE/01 at passage 66 and approximately 120 hours for PE/01 at passage 7. These results might suggest an increased growth rate with passaging but not much change at later passages.

2.2.4 Cell volume

Cell volume measurements were done using an electronic particle size analyser (Coulter Counter Model Z_B with Coulter Channelyzer and X-Y recorder, Coulter Electronics

TABLE 6

COLONY FORMING EFFICIENCY OF PE/01 AND PE/04 IN SUSPENSION IN SOFT AGAR

PE/01			PE/04		
Passage no.	Cell no. in agar	CFE	Passage no.	Cell no. in agar	CFE
2	2×10^4	0.16	2	10^3	1.9
	5×10^4	0.31		2×10^3	2.5
	10^5	0.33		5×10^3	2.2
11	2×10^4	0.35	11	10^3	1.4
	5×10^4	0.34		2×10^3	2.5
	10^5	0.40		5×10^3	2.2
79	10^3	3.1	49	5×10^2	11.3
	2×10^3	10.3		10^3	10.1
	4×10^3	11.2		2×10^3	12.4

Ltd). Latex beads at 12.9um and 14.2um diameter were used as calibration standards and a linear response of the recorder with particle volume was observed. Both cell lines showed a distribution of cell sizes equivalent to a normal distribution with a standard deviation of approximately $\pm$ 400um^3 in volume although there was a small tail to the higher size range. The means of this distribution were calculated and averaged from 5 experiments giving a volume of 1230 $\pm$ 190 (SEM) and 950 $\pm$ 100um^3 for PE/01 and PE/04 respectively. This is equivalent to diameters (assuming spherical cells) of 13.2 $\pm$ 0.7 (SEM) and 12.1 $\pm$ 0.4um for PE/01 and PE/04. While these figures are not significantly different ($p>0.10$) and each cell line showed a spread of cell sizes, the average volume of PE/04 was only 77% of PE/01.

2.2.5 DNA content

Relative DNA content was measured by flow microfluorimetry using normal human lymphocytes as a standard. Cells were harvested by trypsinisation, spun down and resuspended and stained in buffer containing 50ug/ml propidium iodide either by the method of Fried using hypotonic sodium citrate (0.1% sodium citrate, 0.1% triton X-100, J. Fried et al, 1978) or by the method of Taylor simply using 1% triton X-100 in PBS (I.W. Taylor, 1980). Lymphocytes from peripheral blood were resuspended and stained in the same manner, after lysis of erythrocytes with 0.168M NH_4Cl, and

mixed with the ovarian carcinoma cells. Approximately 10^4 cells were analysed on a FACS IV fluorescence activated cell sorter (Becton Dickinson) in the MRC Clinical and Population Cytogenetics Unit operated by Harris Morrison. Both cell lines PE/01 and PE/04 showed a single G_1 peak at slightly greater DNA content than the lymphocytes. This peak was 1.1X diploid in the hypotonic sodium citrate method and 1.3X diploid in the triton X-100 method with no difference between the 2 cell lines. From DNA profiles using the triton X-100 method without a lymphocyte marker, approximately 75% of PE/01 cells and 65% of PE/04 cells were in the G_1 phase of the cell cycle in logarithmic cultures.

2.2.6 Antigenic determinants

PE/01 and PE/04 cultures grown in 75cm^2 flasks (Nunc, Gibco Ltd) were harvested in versene using a cell scraper (Costar) and without any enzyme treatment. The cells were suspended in medium containing foetal calf serum, a drop of human blood added (to add some red blood cells as a control) and the suspension spun down to a cell pellet. Cell smears on clean microscope slides were produced from this cell pellet and frozen at -20^{o}C. The slides were sent in dry ice to Dr. Lynda Bobrow, Department of Histopathology, University College Hospital in London who had a strong collaboration with the I.C.R.F. laboratories. Slides were prepared in this way after discussions with

her and her results with various monoclonal antibodies are shown in Table 7. PE/04 but not PE/01 showed expression of placental alkaline phosphatase, which has been associated with malignant ovarian tumours (J.O. Davies et al 1985). Conversely vimentin was expressed in PE/01 but not in PE/04.

2.2.7 Hormone receptors

Oestrogen and progestogen receptors were kindly assayed by Dr. Tony Hawkins of the Department of Clinical Surgery of the University of Edinburgh using a biochemical assay used for assaying breast cancer biopsies (R.A. Hawkins et al 1981). Seven 75cm^2 flasks of each cell line were grown to confluence and then changed to fresh medium, containing serum stripped of steroids to exclude endogenous oestradiol, for 6 days as recommended by Hamilton (T.C. Hamilton et al 1983b). Serum was stripped of steroids by the method of Stanley using dextran-coated charcoal (E.R. Stanley et al 1977). Cells were harvested to a cell pellet and conveyed to Dr. Hawkins' laboratory at 4^{o}C. The results showed 169 fmol oestrogen receptor sites/mg soluble protein for PE/01 and 219 fmol receptor sites/mg soluble protein for PE/04. When the experiment was repeated, this time after 10 days culture in steroid stripped medium the results were similar with slightly lower levels of 121 and 187 fmol oestrogen receptor sites/mg soluble protein for PE/01 and PE/04 respectively.

TABLE 7

	PE01	PE04
5.2 = monoclonal which recognises low MW cytokeratins viz 8, 18 +19	+++ cytoplasmic granular staining in more than 75% cells.	+++ cytoplasmic granular staining in more than 75% cells.
LP34 = monoclonal which recognises cytokeratin epitopes, which have not been fully characterised. (= very strong expression in squamous carcinoma).	+++ cytoplasmic filamentous network in more than 75% cells.	+++ cytoplasmic filamentous network in more than 75% cells.
AuA_1 = mab against membrane glycoprotein (not fully characterised) present in many epithelia.	+++ membrane staining in more than 75% cells.	+++ membrane staining in more than 75% cells.
Vimentin = mab against the intermediate filament present in mesenchymal cells. It is however, known to be expressed in some epithelial cells in culture and in body fluids.	25-50% cells. +++ cytoplasmic staining.	-
H317 mab against placental alkaline phosphatase (Reagan enzyme types).	-	25-50% cells +++
H17E2 mab against placental alkaline phosphatase. (Non Reagan type)	less than 1% of cells show pale cytoplasmic staining.	More than 50% cells +++.

TABLE 7 (contd)

	PE01	PE04
UJ13a mab raised against human foetal brain. Recognises nervous tissue neroblastomas and neuroendocrine tumours including small cell lung cancer (SCCL)	-	-
123C3 mab raised against SCCL. Similar reactivity to UJ13a.	-	-
B-human chorionic gonadotropin Commercial mab	-	-
∝- fetoprotein. Commercial mab	-	-

Progestogen receptor sites were also assayed in this experiment but could not be detected.

2.2.8 Mer phenotype

A cryotube of cells from each cell line frozen in liquid nitrogen was sent in dry ice to Dr. Tomas Lindahl at I.C.R.F. in London. They were then grown by him and assayed for the O^6-methylguanine-DNA methyltransferase protein. Both were shown to be mer^+ cell lines with 2.77 and 3.29 units methyltransferase/mg protein for PE/01 at passage 83 and PE/04 at passage 55 respectively.

2.3 Other ovarian cancer samples and cell lines

2.3.1 Cell lines related to PE/01 and PE/04

PE/06

As shown in Figure 3 above, a third ascites was obtained from the same patient a week before she died. Many less cells were obtained from this ascites than from the two from which were derived the cell lines PE/01 and PE/04. The cells from the ascites were therefore initially stored in liquid nitrogen and not cultured for over 2 years until the latter part of 1985 when a cell line designated PE/06 was successfully derived. Sub-lines were derived separately from the ascites by culturing with either 5% O_2 or 20% O_2 in the gas phase (5% O_2, 5% CO_2, 90% N_2, high humidity versus 5% CO_2, air, high humidity). At 20% O_2 a

suspension culture was also established. Cytology at passage 2 was similar to that of PE/01 and PE/04. Initial experiments (passages 2 and 4) have shown that the PE/06 subline derived in 5% O_2 had considerably higher colony forming efficiency (in 5% O_2) than the subline derived in 20% O_2 (in 20% O_2) in 6 well plates. Other experiments relating to drug sensitivity are reported in later chapters.

PE/01 - CisPt^R

Late passage PE/01 cells (passage 78) were cultured in 75cm^2 flasks in the presence of low concentrations of cis-platinum starting at 25nM and increasing the dose when the cells appeared unaffected by the preceeding one. The culture medium was replaced every 3 days and fresh cis-platinum added at that time. This resulted in a reasonably close but intermittent dose schedule since the half life of cis-platinum toxic species in medium will only be some hours (A.J. Repta et al 1980). Dose increments were 25nM, 50nM, 100nM, 500nM and 1uM. A dose of 500nM was quickly reached after 6 weeks culture. However it was two-and-a-half months before the dose could be increased to 1uM (at passage 89) and passaging was continued at this dose until passage 107. Cells at different dose levels and passages were stored in liquid nitrogen. This cell line was then passaged for a further 4 months until passage 124 without cis-platinum to see if

it retained its resistance to the drug. No attempt was made to incubate the cells in concentrations of cisplatinum higher than luM. Plated at low density in 6 well plates the colony forming efficiency of 9.9 $\pm$5.7% (passage 102-122) was superior to PE/01 and the colonies formed were tighter and more distinct. In 75cm^2 flasks the line appeared to grow faster than PE/01 although the doubling time has not been formally checked. Measured at passage 110 the line still showed the same DNA content (by flow microfluorimetry) as PE/01 with only one G_1 peak observed and approximately 65% of cells in the G_1 phase of logarithmic cultures. At this time the line also still showed a Mer$^+$ phenotype with 1.64 units O^6-methylguanine-DNA methyltransferase/mg protein. This was slightly lower than the parent PE/01 line (2.77 units) but further work would be needed to test its significance. The cell volume at a mean of 1200 $\pm$ 200um^3 or cell diameter of 13.1 $\pm$ 0.7um was the same as PE/01. Drug sensitivity of the cell line is reported in Chapter 3.

PE/04 - Cis-Pt^R

At the same time as PE/01, late passage PE/04 (passage 48) was cultured with cis-platinum as reported above for PE/01 Cis-Pt^R. While a 500nM cis-platinum dose was reached in the same time higher resistance was more difficult to develop in PE/04 than in PE/01. PE/04 was much slower to become tolerant of the 500nM dose. After 5 months a luM

dose was attempted (at passage 57) but under these conditions the cells grew poorly. A second attempt from an ampoule of cells (passage 57) stored in liquid nitrogen was also unsuccessful. However a third attempt from another ampoule proved more successful. After one passage without drug, 500nM cis-platinum was introduced at passage 59, and lum cis-platinum 7 weeks later at passage 65 and a subline which tolerated this dose eventually produced. At passage 75 further passaging was continued in the absence of drug to see if resistance was maintained.

2.3.2 Other tumour samples and derived cell lines

During this project it became apparent that while interesting conclusions could arise from the PE/01 and PE/04 model and its related samples and sub-lines from the one patient, it would be important to increase the generality of any such conclusions by testing samples from other patients either untreated or undergoing various therapies. I was particularly interested in samples from patients prior to chemotherapy since not only would these provide a benchmark for drug sensitivity in vitro, but there would also be the possibility of obtaining future samples subsequent to therapy allowing valuable comparisons to be made. Since ascites fluid needs to be removed for the comfort and well-being of the patient concerned liaison with the Oncology ward staff of the Department enabled these samples to be collected. Many

such ascites samples were initially stored in liquid nitrogen after initial centrifugation, washing and removal of red blood cells and then the most interesting samples cultured at a later date since this is a time consuming process in the laboratory. The efforts towards deriving cell lines was continued. This cell culture work was a combined effort by various members of the laboratory. Factors such as keeping a varied cell population in initial cultures, high cell density and when to passage cells into larger culture dishes, the use of conditioned medium from other cell lines or the addition of sterile filtered ascites fluid, partial or complete replenishment with fresh medium, differential trypsinisation, recognition of different cell types and in general how well a cell culture is looking are all a matter of experience and everyone in the laboratory contributed to this. I worked with some of the ascites and also cultured cells from the only solid tissue biopsy we received as no one else in the laboratory had experience in culturing cells from solid biopsies rather than from effusions. The samples we received and their culture are summarised in Table 8. Twenty-seven ovarian carcinoma ascites and 3 ascites from patients with adenocarcinomas presumed to be ovarian have now been received and we have attempted to culture 17 of these and 11 cell lines have been derived. Two of these cell lines were not epithelial cells and curiously turned out to be EBNA positive lymphoblastoid

TABLE 8

OVARIAN CANCER SAMPLES COLLECTED

Ovarian ascites	Date collected	Attempt to Establish cell line	Cell line	Patient's treatment
PE/01	18.2.82	Feb. 82	Yes	CisPt, Chb, 5-FU*
PE/02	27.10.82	Oct. 82	No	PredM
PE/03	30.11.82	July 85	No	CisPt, PredM, 5FU, HMM, CTX
PE/04 (same patient as PE/01)	22.12.82	March 83	Yes	As PE/01, and further courses of same treatment
PE/05	1.2.83	-	-	Mustine, Iscador Bleomycin
PE/06 (same patient as PE/01 and PE/04)	3.2.83	Oct. 85	Yes	As PE/04
PE/07	22.3.83	-	-	Chb, CisPt, Spirolactone
PE/08	5.9.83	-	-	Chb
PE/09	19.4.84	April 84	No	Untreated
PE/010 (same patient as PE/09)	27.4.84	April 84 July 84	No	Untreated
PE/011	13.6.84	June 84	No	Thiotepa
PE/012	23.10.84	Oct. 84	No	Chb
PE/013	6.11.84	Nov. 84	Lympo-blastoid line	Untreated
PE/014	4.4.85	April 85	Yes	Untreated
T/014 (same patient as PE/014 but solid tumour biopsy)	16.4.85	April 85	Yes	Untreated
PE/015	29.5.85	May 85	No	Untreated
PE/016	30.5.85	May 85	Yes	Radiotherapy

TABLE 8 (contd)

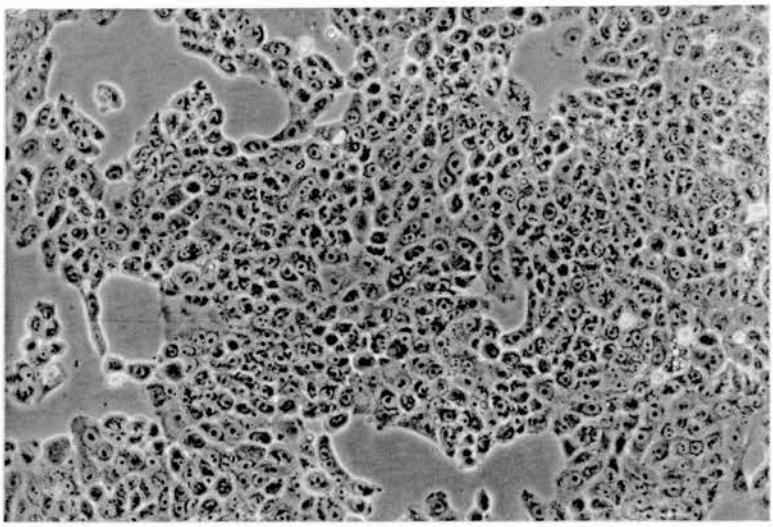
OVARIAN CANCER SAMPLES COLLECTED

Ovarian ascites	Date collected	Attempt to Establish cell line	Cell line	Patient's treatment
PE/017	10.6.85	-	-	CisPt, 5-FU, HMM, PredM, Chb
PE/018	20.6.85	-	-	Untreated
PE/019	2.7.85	-	-	Untreated
PE/020 (same patient as PE/017)	5.8.85	-	-	As PE/017
PE/021	5.9.85	-	-	Untreated
PE/022	11.9.85	-	-	CisPt, 5-FU, HMM, PredM
PE/023 (same patient as PE/014)	22.10.85	Oct. 85	Yes	CisPt, PredM (after PE/014)
PE/024	26.11.85	-	-	CisPt, 5-FU, HMM, PredM, Chb, Mitozolamide
PE/025 (same patient as PE/024)	3.12.85	-	-	As PE/024
PE/026	22.1.86	-	-	Untreated
PE/027	31.1.86	-	-	Chb
Ascites from adenocarcinoma presumed ovarian				
PE/A1	13.9.82	Sept. 82	Yes	Untreated
PE/A2 (same patient as PE/A1)	14.3.83	June 85	Yes	CisPt, Chb
PE/A3	21.3.85	March 85	Lympo-blastoid line	Untreated

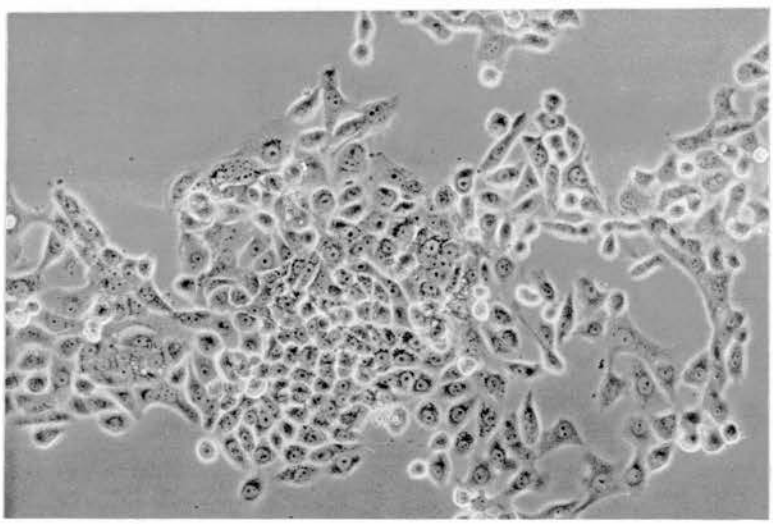
* CisPt - cisplatinum, Chb - chlorambucil, 5-FU, 5-fluorouracil, PredM - prednimustine, HMM - hexamethylmelamine, CTX - cyclophosphamide

cell lines. PE/014 and PE/023 were derived from the same patient before and after treatment although the patient showed no response to her chemotherapy. The cell lines PE/A1 and PE/A2 were similarly derived from ascites before and after treatment although this patient's cancer was an adenocarcinoma presumed but not positively identified to be ovarian. The cell line T/014 which I derived showed similar cytological characteristics and growth patterns in vitro to PE/014 derived from ascites from the same patient at the same time. It was established by mechanical disaggregation of the biopsy and removal of the fatty tissue, culture in RPMI 1640 plus 10% foetal calf serum and with differential trypsinisation to prevent fibroblast overgrowth of the initial epithelial colonies. In dense cultures T/014 and PE/014 showed a marked tendency to form domes above the growth surface which grew into rounded cysts. Four parallel cell lines and a cell line which grew as cell clusters in suspension were derived from the T/014 solid biopsy. Characterisation of various antigenic phenotypes of these samples and cell lines has been done by Dr. Francis Hay and Dr. Simon Langdon. Only 2 of the patients from whom an ascites sample was obtained before treatment (PE/015 and PE/026) are still alive. Examples of cell lines PE/01, PE/04, PE/014 and PE/016 in culture on plastic are shown in figures 12 and 13.

Figure 12 Ovarian Carcinoma cells in Tissue Culture



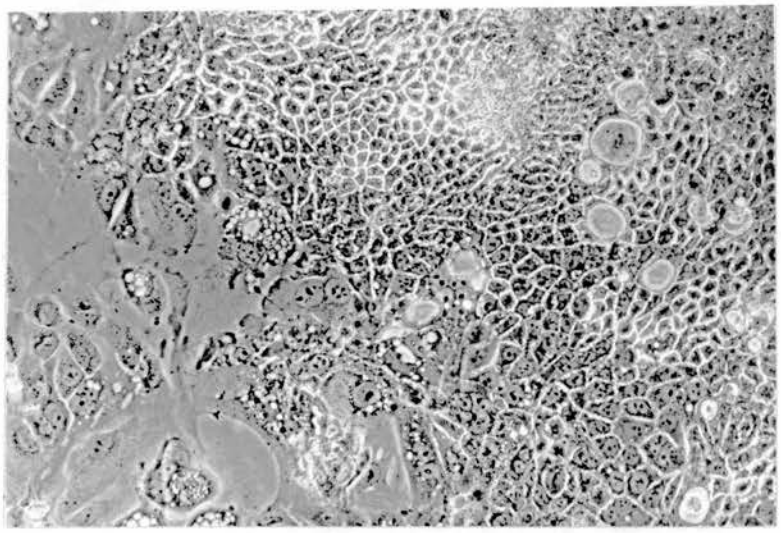
PE/01 passage 77
(mag. x425)



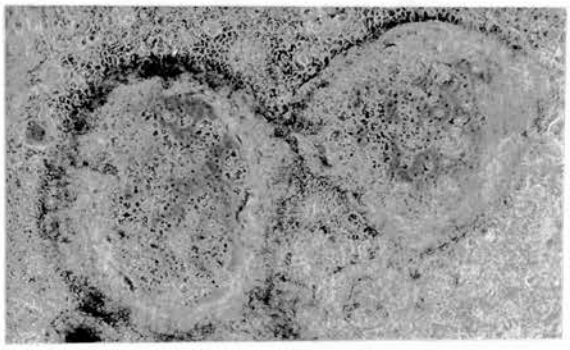
PE/04 passage 49
(mag. x425)

Figure 13 Ovarian Carcinoma cells in Tissue Culture

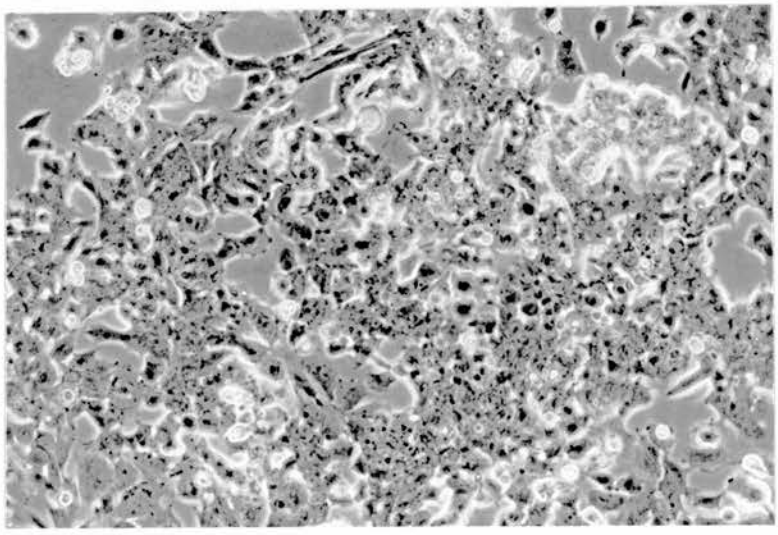
PE/014
passage 10
(mag. x425)



Showing formation
of rounded cysts
(mag. x100)



PE/016
passage 10
(mag. x425)



2.4 Discussion

These characterisations are consistent with the published literature on ovarian carcinoma cell lines as referred to in chapter 1. Recent studies (A.P. Wilson 1984; I. Bertoncello et al 1985; R.N. Buick et al 1985; J. Benard et al 1985) have shown similar cytological and ultrastructural features and similar results in establishing and growing ovarian carcinoma cells in vitro. PE/01 and PE/04 appear cytologically similar to each other and to the 2 ascites from which they were derived and do not show any separate morphologically distinct subpopulations. The different types of cells observed would appear to derive from each other depending on culture conditions in a similar way to that observed by Whitehead for a breast adenocarcinoma cell line (R.H. Whitehead et al 1983). More of the differentiated type cells were observed in initial cultures than in later passages when growth was more rapid. Features at the ultrastructural level were consistent with the light microscopy and with other ultrastructural studies of epithelial ovarian tumours (C.M. Fenoglio, 1980).

The cytogenetic analysis for PE/01 and PE/04 gives some indication of the relationship between the 2 cell lines and their possible evolution. The cell lines have several rearranged chromosomes in common and some distinct differences particularly normal chromosomes 8 and 17 in

PE/04 but not PE/01, suggestive of a common ancestor with subsequent divergence but not lineal descent from PE/01 to PE/04. The DNA content of both cell lines was slightly greater than that of a normal human cell suggesting that within the hypodiploid karyotype are rearrangements incorporating additional DNA. Only one near diploid peak of DNA was observed by flow microfluorimetry in late passage cells of PE/01, PE/04 and the resistant subline PE/01 CisPt^{R} suggesting there had not been the changes towards higher aneuploidy during culturing observed by others (C. van Haaften-Day et al 1983). If the cell lines are representative of the ascites from which they were derived this analysis would support the notion of a pre-existing drug resistant sub-population (represented by PE/04) which is simply selected by treatment. Buick and his colleagues have described antigenic, growth and karyotypic variations in 5 cell lines. Two of these cell lines were from the one patient from two ascites 9 months apart that were karyotypically similar. The first cell line was karyotypically different but remained stable in culture while the second line represented a karyotypically minor population in the ascites but showed marked changes in culture (J.M. Trent et al 1985). Horowitz has shown selection for particular cells (carcinoembryonic antigen positive) in primary culture (A.T. Horowitz et al 1985). Both Benard and Wilson have studied features at different culture passages without noticing significant

morphological or cytogenetic changes, although Wilson noted some changes in chemosensitivity. Bertoncello noticed varying CFE amongst clones from the same and different tumours and some variation within the basic karyotype of a family of clones. Clearly conclusions about a patient's tumour drawn from an analysis of derived cell lines can only be tentative but the results for PE/01 and PE/04 suggest a heterogeneous tumour.

PE/04 is a little more robust in culture than PE/01 with more prolific primary cultures and a higher colony forming efficiency at low cell density at all passages but cell doubling time and bulk culture in flasks were similar. It is difficult to know why the CFE for late passage PE/04 but not PE/01 should be higher in soft agar than on plastic growth surfaces. Presumably more work to optimise the growth conditions in either case could improve the CFE. Perhaps the low oxygen tension is a factor or it may be that PE/04 is more prone to grcwth in suspension than PE/01 as evidenced by the establishment of a suspension culture cell line from its ascites in addition to the monolayer cell line used above.

The PE/01 $CisPt^R$ resistant subline was derived with comparative ease. Whether the difficulty in producing the PE/04 $Cis\text{-}Pt^R$ resistant subline was simply due to laboratory mistakes (e.g. wrong dilution of the cis-

platinum) or caused by some innate property of the PE/04 cell line is not completely clear. However it is tempting to suggest that PE/04, being already more resistant to cis-platinum than PE/01 (see chapter 3), found it more difficult to produce further resistance. Certainly the mechanisms of resistance of the various lines will prove interesting. With both cell lines were stopped at 1uM cis-platinum since the LD50s of the parent lines were 0.08uM and 0.2uM cis-platinum for PE/01 and PE/04 respectively (data in chapter 3) and thus sub-lines which grew in 1uM cis-platinum must be significantly resistant.

Other features of PE/01 and PE/04 such as their antigenicity were fully consistent with cells from an epithelial ovarian adenocarcinoma. The most interesting antigens were those showing differences between the 2 cell lines (placental alkaline phosphatase and vimentin) and these differences could potentially be useful in distinguishing the lines from each other in experiments where they were mixed together. Later work by Dr. Francis Hay and Dr. Simon Langdon has extended the range of antibodies tested on these lines and other ovarian ascites samples and cell lines in this laboratory. One difference in their work was a low level of antibody staining with vimentin in both PE/01 and PE/04, although vimentin content can change with culture conditions (N.D. Connell et al, 1983).

The oestrogen receptor levels in PE/01 and PE/04 are very high compared with those usually observed in ovarian carcinomas (M. Lantta 1984; J.S. Lazo et al 1984) or in the only other ovarian cell line reported (T.C. Hamilton et al 1983b). The relative proportions of oestrogen and progestogen receptors reported were variable but included oestrogen receptor positive/progestogen receptor negative tumours. It has also been shown that oestrogen (specifically excluded in the protocol here) can induce the production of progesterone receptors (P.G. Satyaswaroop et al 1983; T.C. Hamilton et al, 1984b) and it may be that hormone influences are important in the PE/01 and PE/04 cell lines.

In cell lines derived from other human tumours other authors have noted stable characteristics of cell lines over long periods in culture (W. Tilgen et al 1983) and also that consistent features of the parent tumour are present (K.M. Tveit 1981a). Various morphological features in cell lines from different carcinomas are also common to the cell lines described here and include not only the characteristic shape of epithelial cells but also cells full of lipid as in breast cancer cells (P.S. Rudland et al 1985) or the formation of domes and cysts as in colon adenocarcinoma (S.C. Kirkland 1985). Further characterisation of the various ascites samples and cell lines of ovarian carcinomas is continuing in this

laboratory. This project concentrated on PE/01 and PE/04 and these 2 cell lines were compared for more specific features related to drug metabolism and sensitivity as described in the succeeding chapters.

3. PE/01,PE/04 AND RELATED CELL LINES - SENSITIVITY TO ANTINEOPLASTIC DRUGS

In this chapter data on drug sensitivity testing in the cell lines PE/01, PE/04, PE/01 CisPt^R and preliminary data for PE/04 CisPt^R and PE/06 are described. Unless otherwise indicated assays have been done at the later passages, 77-82 for PE/01 and 46-51 for PE/04. Initial work concentrated on the drugs used clinically viz cisplatinum, 5-fluorouracil and chlorambucil. Later work tested other drugs for cross-resistance.

Two different assays have been used. When I joined the Unit a Courtenay assay (see below) had been set up but difficulty was being experienced in obtaining consistent results due to various technical problems with the assay. Since the data were preliminary and it was imperative to be sure what differences in drug sensitivity there were between PE/01 and PE/04 and how large these differences might be, I set about establishing a new assay which might be less troublesome, but still reliably measure clonogenic cell survival. With cell lines it is not necessary to use agar to inhibit the growth of the fibroblasts found in primary samples. Colony forming efficiency is also usually higher. Therefore assays where cells are plated directly onto a plastic growth surface are commonly used with cell lines (R.T. Hill, 1983) and I had previous experience with such an assay (P.G. Parsons et al, 1982).

The ovarian cell lines here all formed discrete colonies on plastic which could be counted by microscope and so the clonogenic assay on plastic (see below) later became the routine drug sensitivity assay in this laboratory. The relative merits and problems with these different assays are discussed below.

3.1 Methods

3.1.1 Courtenay assay

The protocol of Courtenay (V.D. Courtenay and J. Mills, 1978; V.D. Courtenay et al, 1978) was followed in detail. This assay was preferred to that of Hamburger and Salmon (A.W. Hamburger and S.E. Salmon, 1977) since it shows higher colony forming efficiences (K.M. Tveit et al, 1981).

Cells in logarithmic growth were harvested by trypsinisation, resuspended in Hams F12 medium + 10% foetal calf serum (Gibco), and a single cell suspension obtained by vigorous pipetting or passing through a 19 gauge needle. Cell counts, clumping and viability were assessed by counting nigrosin (Sigma) stained cells in a haemocytometer. Cells were suspended at 5x the final concentration required (5 x 10^3 and 10^3 cells final concentration for PE/01 and PE/04 respectively). Red blood cells were obtained from anaesthetised August rats by cardiac puncture with a heparinised syringe, the buffy

coat removed by centrifugation, the cells washed in phosphate buffered saline and made up to the original volume in Hams F12 medium + 10% foetal calf serum. 5% agar (Agar Noble, Difco Laboratories) in water was prepared by autoclaving, cooled to 44°C and diluted to 0.5% with medium prewarmed to 37°C. 3.6mls of this agar was added to a mixture of 1.2mls of the 5x cell suspension, 0.6mls of a 1 in 8 dilution of the August rat red blood cells (less than 3 weeks old stored at 4°C) and 0.6mls of drug in medium at 10x final concentration. 1ml aliquots were pipetted into 5 replicate tubes (Falcon 2051) and the tubes placed in crushed ice to set the agar. Each tube was then gassed for 20 seconds with the sterile filtered gas mixture (5% O_2, 5% CO_2, 90% N_2), placed upright in a plastic box and then the box gassed, sealed and placed in an incubator at 37°C. Later in this project when an incubator with this gas mixture was obtained (Hereaus, model B5060 EK/O_2) this gassing step could be omitted. After a week 1ml of medium was added to the tubes and again thereafter at weekly intervals, the tubes being regassed on each occasion. When colony size was greater than approximately 50 cells, usually 21 days, the agar plug was removed onto a petri dish lid. The grided bottom of the petri dish was used to flatten out the agar and assist visual counting of colonies at 40x magnification using a Leitz Diavert inverted microscope. Clonogenic survival was measured as the percentage of

control after counting colony numbers in drug treated and control untreated tubes. Any variation from this procedure is discussed with the particular experiment involved.

3.1.2 Clonogenic assay on plastic

Cells were plated out in 6 well plates (Linbro, Flow Laboratories) in 2mls medium (RPMI 1640 plus 10% foetal calf serum with added insulin (2.5ug/ml), sodium pyruvate (1mM), antibiotics streptomycin (100ug/ml) and penicillin (100 IU/ml) and buffered with up to 12.5mM MOPS) at a cell density to give approximately 100-200 colonies per well (PE/01 2-3 x 10^3/ml, PE/04 10^3ml, PE/01 CisPt^R 0.5-3 x 10^3/ml) taking care to have a single cell suspension. Incubation was at 37^oC in a 5% CO_2, high humidity incubator (Vindon Scientific Ltd). The next day 1ml extra medium was added. The medium was changed on the third day and drugs were added in fresh medium to wells in triplicate. The cells had attached to the plastic growth surface but not divided at this stage. Three days later the medium with drug was removed and replaced with fresh medium which was changed every 2 to 3 days thereafter. Colonies of 50 cells or more were counted (by eye at 40x magnification) 10 to 12 days after addition of the drug and then pulsed with 1uCi/ml [methyl-^{3}H]-thymidine (Amersham) for 3-5 hours before being harvested by trypsinisation and washed on to glass fibre filters (GF/A,

Whatman) with 5% trichloroacetic acid. These filters were then dried and counted on a Packard Tri-Carb 4000 liquid scintillation counter with Unisolve E (Koch-Light Ltd) as the scintillant. Counts were expressed as a percentage of control untreated wells and dose response curves drawn on semi-log plots.

Counting colonies by eye and by a [^{3}H]-thymidine pulse at the same time to measure dose response showed good agreement (see results below). The [^{3}H]-thymidine pulse and measurement of label incorporated into TCA precipitable material (i.e. DNA) should be a relative measure of the number of growing cells per well and hence the colony number when done when cells have had time to fully recover after drug treatment. In later experiments only 1 well per triplicate (usually the central wells of a 6 well plate) was counted visually to check that the radiolabelling method did not give artifactual results in any particular experiment.

Early experiments used Sterilin 6 well plates (Sterilin, U.K.) but some variation in CFE between wells and the occasional well where cells did not attach led us to check plates of other suppliers. Nunc 6 well plates (Nunc, Gibco Ltd) gave poorer CFE perhaps due to the greater depth of the wells in the plates. Both Linbro plates (Linbro, Flow Laboratories) and Falcon plates (Becton

Dickinson and Co.) gave good results. Most experiments were done using the Linbro 6 well plates.

3.1.3 Antineoplastic drugs used

Most drugs were obtained from the same sources as used by the clinical staff in the Department and made up as per the manufacturers instructions before diluting in medium. Cisplatinum was from Farmitalia Carlo Erba Ltd (with sodium chloride and mannitol) and the powder was reconstituted with water to a concentration of 5mM. Aliquots of this stock were kept frozen at -20°C. 5-fluorouracil was from Roche Products Ltd. in saline at 25mg/ml. Chlorambucil and melphalan were from Sigma Chemical Co. Ltd. and were dissolved in dimethylsulphoxide (10mM) or in 0.1N HCl (10mg/ml) respectively immediately before use. Some melphalan (as "Alkeran") was also obtained from the Wellcome Foundation. The platinum analogues CBDCA and CHIP were a kind gift from Dr. M. Jones and Professor K.R. Harrap of the Institute of Cancer Research, Sutton and were dissolved in saline (Dulbecco's phosphate buffered saline) at 5mM concentration and aliquots frozen as a stock solution. JM40 was from Bristol-Myers Pharmaceuticals as a solution at 5mg/ml and was also diluted in saline to 5mM and aliquots frozen. Doxorubicin was from Farmitalia Carlo Erba Ltd ("adriamycin" with lactose) and was dissolved in water at 10mM and aliquots of this stock solution frozen.

Vincristine (as "oncovin" with lactose) from Eli Lilly and Co. Ltd. was dissolved in saline to 1mM and aliquots frozen. Mitozantrone was from Lederle Laboratories as a 2mg/ml solution ("Novantrone"). Prednimustine, a gift from Aktiebolaget Leo, (Helsingborg, Sweden) was dissolved at 10mM in DMSO and used immediately. Experiments using X-rays were done using a Siemens Stabilipan 2 X-ray set with a Farmer dosimeter type 2570 (Nuclear Enterprises Ltd) at a dose rate of approximately 35 rads/minute.

3.2 Results of assessment of assay conditions

3.2.1 Courtenay assay

It was decided to continue with this assay for two reasons. Firstly the preliminary data was suggestive of some differences in drug sensitivity between the two cell lines PE/01 and PE/04 and it was clearly important to obtain firm data to follow this up. Also this data could then be compared with that of other authors since agar clonogenic assays have come to be regarded as the bench mark for other drug sensitivity assays and are widely used (P.P. Dendy et al, 1983). Secondly as this project developed it was hoped to check back to the drug sensitivity of ascites samples where an agar assay would be necessary to exclude fibroblast growth and Courtenay had shown good growth of tumour cells from ovarian ascites (V.D. Courtenay et al, 1978).

Before I jcined the Unit experiments using PE/01 at from 10^3 up to 10^5 cells per ml showed CFE varying from 0-8.4% with the highest CFE obtained at 10^5 cells/ml giving thousands of colonies. 10^5 cells/ml gave high CFE cn 3 cccasions (7.0, 1.5 and 8.4% CFE respectively) but only 0.1% CFE on a 4th occasicn. At passage numbers between 9 and 45 using between 1 and 5 x 10^4 cells/ml CFE was 0.11 $\pm$ 0.08% (mean $\pm$ standard deviation of 6 experiments) giving too few cclonies for accurate dose response data. This variaticn cculd possibly be due to variation in the growth potential of PE/01 at various times, cr to different batches cf foetal calf serum or rat red blcod cells. In addition scme prcblems with contamination had been experienced thought tc be due to contaminated rat red blcod cell preparations.

After I joined the Unit a concerted effort was made tc clear up these problems. Colony forming efficiency of different passages was checked in cne experiment (at the same time in the same medium) as shown in Table 9 and appeared to be improved at late passage in both cell lines (See also Table 6 in chapter 2 which shows data cn different passages cf these cell lines from an experiment done much later in this project when the assay appeared to be working better). Over all the experiments on the late passage cells using 10^3 up to 5 x 10^3 cells/ml (PE/01 usually 5 x 10^3 cells/ml and PE/04 usually 10^3 cells/ml)

TABLE 9

CCLONY FORMING EFFICIENCY IN AGAR AT DIFFERENT PASSAGES

PE/01			PE/04		
Passage	cell nc. in agar medium	CFE (%)	Passage	cell no. in agar medium	CFE (%)
4	10^3	0	7	5×10^2	1.5
	10^4	0		10^3	1.2
	10^5	0.007		5×10^3	0.75
30	10^3	0.02	26	5×10^2	7.4
	10^4	0.018		10^3	6.3
	10^5	0.087		5×10^3	4.4
78	10^3	0.6	48	10^3	8.9
	10^4	1.2		5×10^3	11.4
	10^5	3.3			

CFE were 3.9 ± 2.5% and 18.3 ± 10.4% for PE/01 and PE/04 respectively (see also my comments on CFE in chapter 2). To overcome the contamination problem we tested the use of rat red blood cell lysate which could be filter sterilised instead of whole red blood cells, as a cell lysate had also been used in the original work using erythrocytes to enhance colony growth (I. Bertoncello et al, 1977) and has been used by others in agar clonogenic assays (J.W. Sheridan et al, 1981). However this appeared to give reduced colony forming efficiency with our cell lines and the use of whole rat red blood cells was continued with extra care taken to obtain the blood sterilely (shave thorax of rat, spray with methylated spirits, only one attempt to withdraw blood, and the procedure done in a laminar floow hood). Courtenay has reported that lysis of the rat red blood cells occurs after about 5 days in the agar culture and this timing is important to the protocol, with August rat red blood cells giving superior results to either cell lysate or cells from Wistar or Marshall rats which did not lyse in the first week of the agar culture (V.D. Courtenay and J. Mills, 1978).

Cell density appeared not to affect the results as similar drug survival curves were obtained with as low as 20 or as high as 4000 colonies in controls. However experiments with very low or very high colony numbers like this were usually excluded from the drug sensitivity analysis since

the accuracy of the percentage survival figures obtained from such experiments is always more doubtful. In most experiments I aimed for approximately 200 colonies in controls. Meyskens has described the importance of cell number plated in the Hamburger/Salmon assay and its effects on drug sensitivity assays (F.L. Meyskens et al, 1983). This may be less important in the Courtenay assay where the medium is replenished during the assay. For PE/04 colony forming efficiency (CFE) was fairly constant down to low cell density but there was a drop off in CFE at low cell numbers with PE/01 but this did not affect the drug survival assay results which are reported as percentage of control.

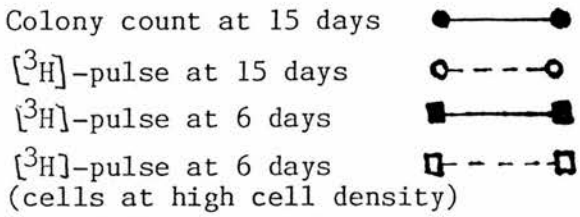
3.2.2 Clonogenic assay on plastic

Initial experiments to check colony forming efficiency and growth in 35mm wells of 6 well plates are described in chapter 2. Two other parameters were important. Firstly the [^{3}H]-thymidine incorporation prccedure, designed to reduce time spent in manual counting at a microscope, needed to correlate well with the colony counts by eye. It might also reduce the time of the assay. Secondly any possible effects of cell density needed tc be determined since it was apparent that at low cell density the colony forming efficiency could increase with cell number.

Three sets of 6 well plates of each cell line were set up,

1 plated at high cell density (6 x 10^4 cells and 3 x 10^4 cells/well for PE/01 and PE/04 respectively) and 2 at low cell density (10^4 cells and 3 x 10^3 cells for PE/01 and PE/04 respectively). Various doses of cisplatinum were added on day 3 and left on for 2 days before being removed and replaced with fresh medium. Six days after the drug treatment the control wells of the high cell density plates started to become crowded with cells and these plates were pulsed with [^{3}H]-thymidine while still in logarithmic phase together with 1 set of the low cell density plates. When colonies of 50 cells or more had grown up in the other set of low cell density plates (after 15 days) the colonies were counted by eye and then pulsed with [^{3}H]-thymidine. The results are shown in Figures 14 and 15. The colony counts and [^{3}H]-thymidine pulse after 15 days correlate well. The correlation of the [^{3}H]-thymidine pulse after 6 days with the 15 day results was not as good particularly at the high drug doses but little difference between the low cell and high cell density plates at this time could be observed. This tendency towards resistance at shorter times appeared even greater in 2 preliminary experiments pulsed at 4, 5 and 7 days and I therefore kept the assay with the [^{3}H]-thymidine pulse done at the time when colonies would normally be counted visually. In all later experiments the correlation at this time between colony counting and [^{3}H]-thymidine pulse was very good.

Figure 14 Timing of [^{3}H]-thymidine pulse in Clonogenic Assay PE/01



Dose (uM)	% Survival			
	Low cell density			High cell density
	Colony count 15 days	[^{3}H] pulse 15 days	[^{3}H] pulse 6 days	[^{3}H] pulse 6 days
0.06	72.1 ± 20.2(a)	83.3 ± 26.5	51.2 ± 17.4	126.5 ± 20.7
0.1	30.2 ± 13.6	30.3 ± 13.1	29.6 ± 9.7	79.4 ± 11.4
0.2	19.8 ± 6.2	16.8 ± 5.1	33.7 ± 10.3	40.3 ± 6.4
0.6	0(b)	?1.1(c)	9.0 ± 2.6	8.8 ± 2.2
1.5	0	?1.0(c)	3.4 ± 1.1	1.7 ± 0.2

(a) standard error of the mean from triplicates

(b) no colonies in all 3 wells. If there was 1 colony per 3 wells then %survival would be 0.4% which therefore represents the limit of sensitivity of the assay

(c) 155-191 cpm against background thought to be 100 cpm ie may be simply a background reading since background was calculated from a well with [^{3}H]-thymidine but without cells ever being in the well.

Figure 14

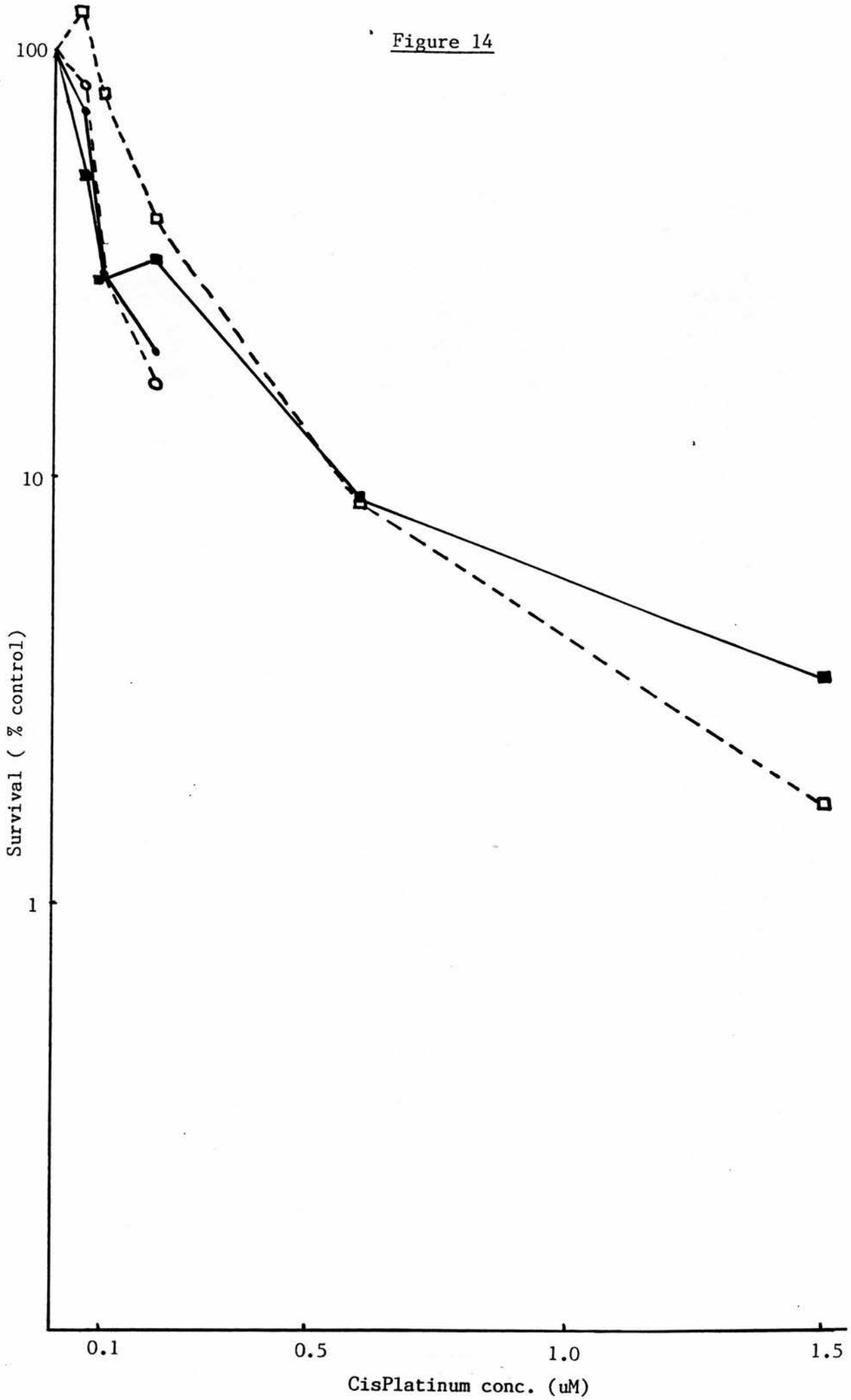
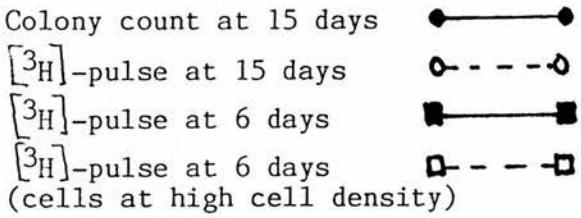


Figure 15 Timing of [^{3}H]-thymidine pulse in Clonogenic Assay PE/04



Dose (uM)	% Survival			
		Low cell density		High cell density
	Colony count 15 days	[^{3}H] pulse 15 days	[^{3}H] pulse 6 days	[^{3}H] pulse 6 days
0.06	96.6 ± 29.9(a)	-(b)	63.0 ± 14.9	112.9 ± 11.4
0.1	50.0 ± 23.4	31.4 ± 20.3	130.3 ± 41.5	102.2 ± 9.5
0.2	51.0 ± 19.1	36.6 ± 19.4	56.7 ± 19.9	68.3 ± 6.8
0.6	14.6 ± 7.6	14.3 ± 9.6	11.7 ± 3.5	31.8 ± 4.0
1.5	0(c)	?1.2(d)	3.8 ± 0.8	2.5 ± 0.5

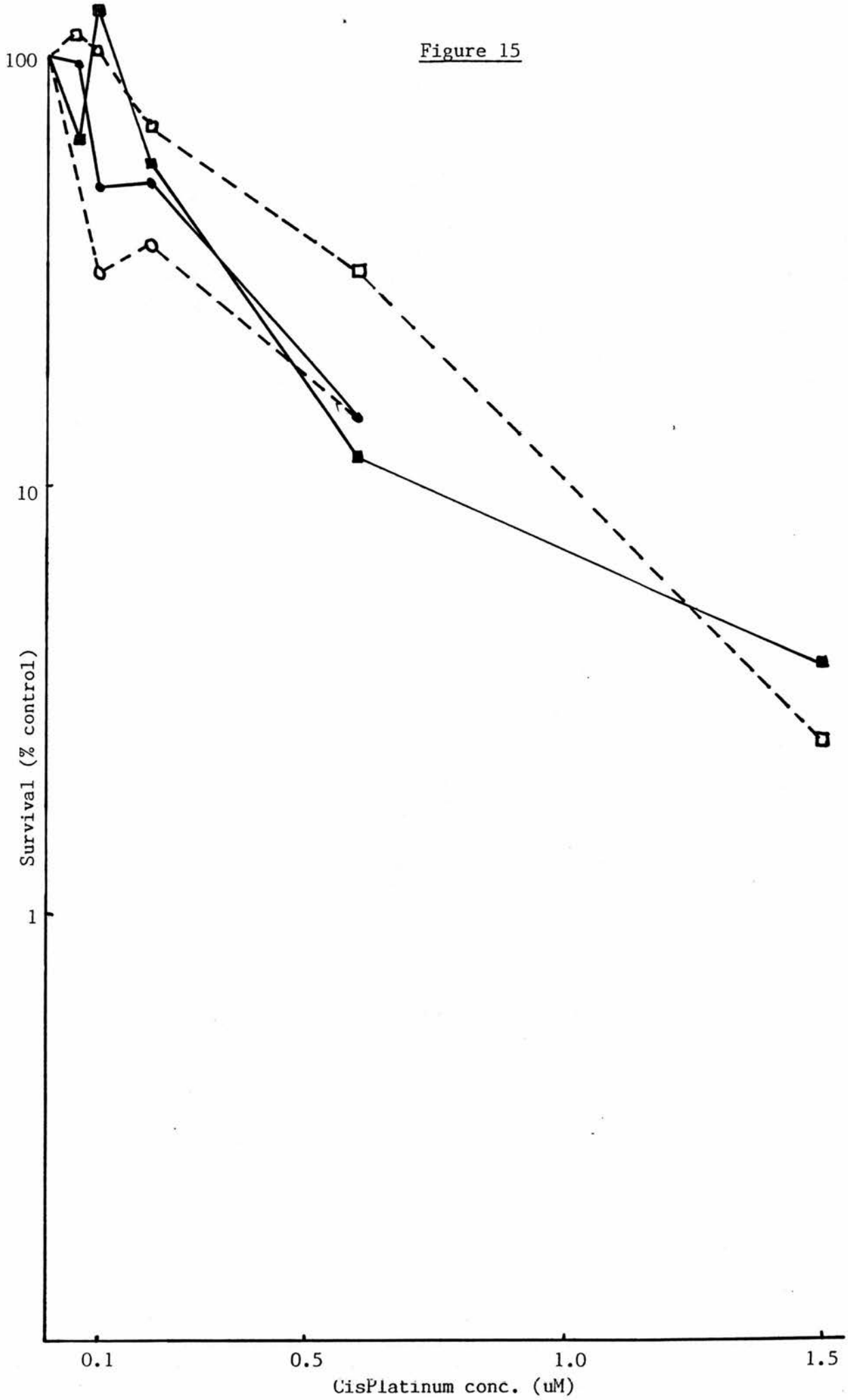
(a) standard error of the mean from triplicates

(b) cells lost during harvesting

(c) as in figure 14, limit of sensitivity approximately 0.4%

(d) 140-169 cpm against background thought to be 100 cpm
ie may be simply a background reading

Figure 15



Cell density did not apparently affect the drug assay results. In the normal assay where I aimed for 100-200 colonies in untreated wells, a number of assays by chance gave greater or less than these colony numbers. These were not discarded unless there were too many colonies to count or too few to give an indication of dose response. Different assays gave the same result across a 10-fold range in colony numbers per well in control wells (i.e. approximately 50-500 control colonies counted). Apparently after the cells have attached as single cells to the growth surface a subsequent reduction in their density does not affect their colony forming efficiency.

3.3 In vitro sensitivity of drugs used clinically

3.3.1 Cisplatinum

Courtenay assay

Combined results from 2 experiments for PE/01 and 6 experiments for PE/04 are shown in Figure 16. Other experiments with PE/01 were consistent with these results but showed fewer than 30 colonies in control tubes or in one instance greater than 4000 colonies in controls. PE/01 is approximately 3 times more sensitive to cisplatinum then PE/04. No variation with passage number was observed.

Clonogenic assay on plastic

Combined results from 8 experiments for PE/01 and 5

Figure 16 CisPlatinum Sensitivity - Courtenay assay

PE/01 ✕——✕

PE/04 ●- - - -●

Experiments at passage 53 and 79 for PE/01 and 7,19,26,33,49 and 50 for PE/04.

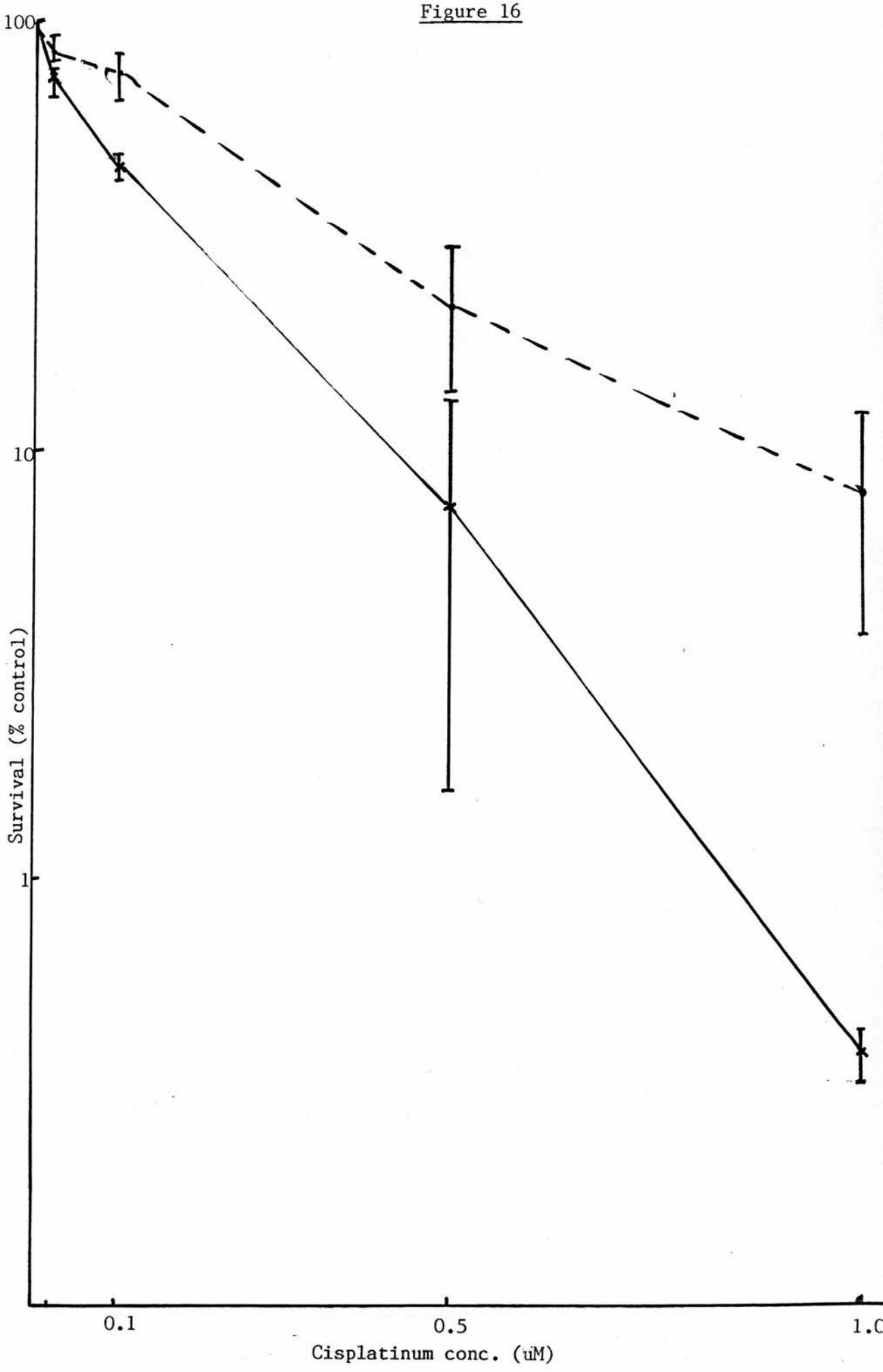
Dose (uM)	% Survival[a] PE/01	PE/04
0.01[c]	87.5 ± 7.2(1)[b]	80.1 ± 12.9(4)
0.02	74.4 ± 6.6(1)	85.4 ± 7.7 (2)
0.1	46.1 ± 3.2(2)	76.2 ± 10.7(6)
0.5	7.4 ± 5.8(2)	21.8 ± 8.3 (5)
1.0	0.39± 0.06(1)	7.9 ± 4.2 (4)
2.0	0.02± 0.02(2)	0.38± 0.24(5)

(a) % survival indicates colony number in drug treated tubes as a percentage of colony number in control tubes.

(b) Standard error of the mean is shown. Number of experiments from which the mean and standard error were calculated is shown in brackets. Where the mean from one experiment is shown the standard error is calculated from 5 replicates.

(c) Dose of cisplatinum was left on for the duration of the assay

Figure 16



experiments for PE/04 are shown in Figure 17. Also shown are the combined results from 6 experiments with the PE/01 CisPt^R line. Figure 18 shows there was no difference between PE/01 CisPt^R at passage 109, (4 experiments), grown in 1uM cisplatinum before testing, and at passages 120-122 (2 experiments), grown without cisplatinum for approximately 3 months since passage 108. PE/01 is approximately 3 times more sensitive to cisplatinum than PE/04, as in the Courtenay assay above, and the PE/01 CisPt^R line is some 25-fold more resistant than its parent line.

Some experiments were also done using 1 hour or 6 hour exposures of cisplatinum since a decrease in toxicity with these shorter exposure times has been observed with this drug (H.T. Rupniak et al, 1983a). One experiment with 1 and 6 hour exposures in the Courtenay assay did show increased resistance with these shorter exposures. However the extra manipulations involved could lead to increased variability due to extra dilutions and the additional procedure of holding cells in suspension, whereas various exposure times are simply achieved in the plastic assay as the drug can be easily removed from the cells attached to the growth surface of a culture well. Therefore other assays were done by this latter method. Figure 19 shows results from 4 experiments for a 1 hour exposure to cisplatinum. The difference in sensitivity of

Figure 17 CisPlatinum Sensitivity - Clonogenic assay on plastic

PE/01 ×——×

PE/04 ●- - -●

PE/01 CisPt^R □— — —□

(Dose of Cisplatinum left on for 3 days)

Dose (uM)	% Survival PE/01	PE/04	PE/01 CisPt^R
0.02	78.6 ± 6.1 (5)(a) (77.2)(b)	-	-
0.06	67.5 ± 10.8(6) (65.4)	60.7 ± 1.9(2) (116.8)	-
0.1	39.2 ± 11.2(7) (50.0)	56.7 ± 9.0(4) (70.3)	-
0.2	17.8 ± 5.8 (7) (16.7)	55.3 ± 8.1(4) (55.1)	-
0.6	2.4 ± 1.0 (7) (0.9)	18.8 ± 4.6(4) (14.1)	99.5 ± 11.4(5) (132.6)
1.0	-	6.2 ± 1.1(3) (2.4)	93.1 ± 10.8(6) (104.1)
1.5	-	1.1 ± 0.2(2) (0)	-
2.0	-	-	31.6 ± 2.9 (5) (58.0)
4.0	-	-	5.0 ± 1.6 (5) (6.0)
6.0	-	-	0.6 ± 0.4 (5) (0.7)

(a) results using the [^{3}H]-thymidine method for calculating survival after drug exposure are shown. The number of experiments from which mean ± standard error were calculated is shown in brackets.

(b) calculated mean from results where colonies were counted visually is shown in brackets (usually only 1 well per triplicate counted).

Figure 17

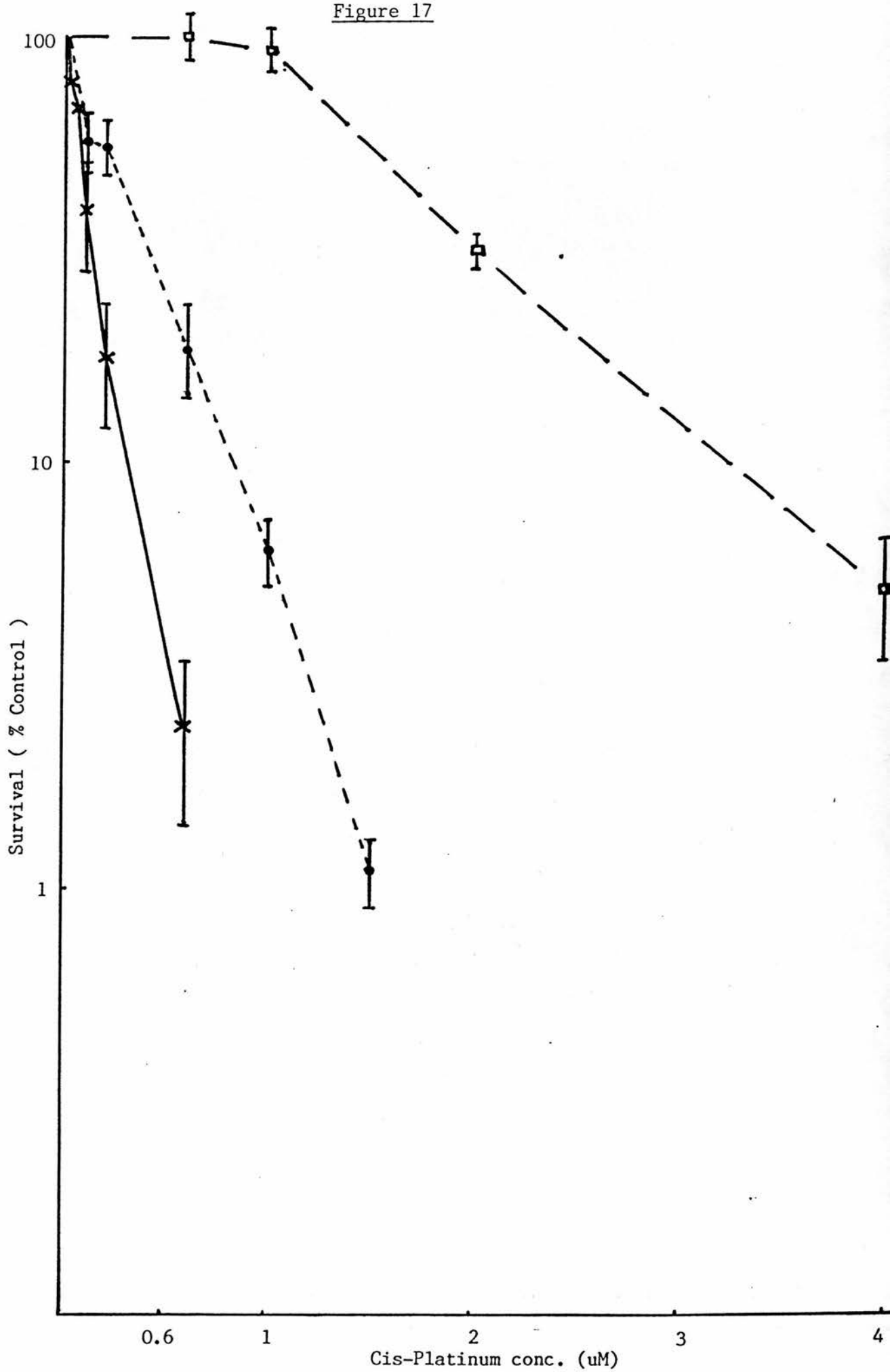


Figure 18 PE/01 CisPt^R - passaged without drug in medium
CisPlatinum Sensitivity

PE/01 CisPt^R passage 109 ✕——✕

PE/01 CisPt^R passages 120-122 ●- - -●

(dose of Cisplatinum left on for 3 days)

Dose (uM)	% Survival[a] PE/01 CisPt^R passage 109	PE/01 CisPt^R passage 120-122
o.6	97.8 ± 16.1 (3) (111.1)	102.0 ± 22.5 (2)
1.0	84.8 ± 10.9 (4) (99.9)	109.6 ± 25.0 (2) (112.5)
2.0	30.6 ± 2.1 (3) (66.4)	33.1 ± 8.3 (2) (45.6)
4.0	4.3 ± 1.8 (3) (6.4)	6.1 ± 3.8 (2) (5.3)
6.0	0.8 ± 0.6 (3) (0.9)	0.4 ± 0.4 (2) (o.4)

(a) notes as in figure 17

Figure 18

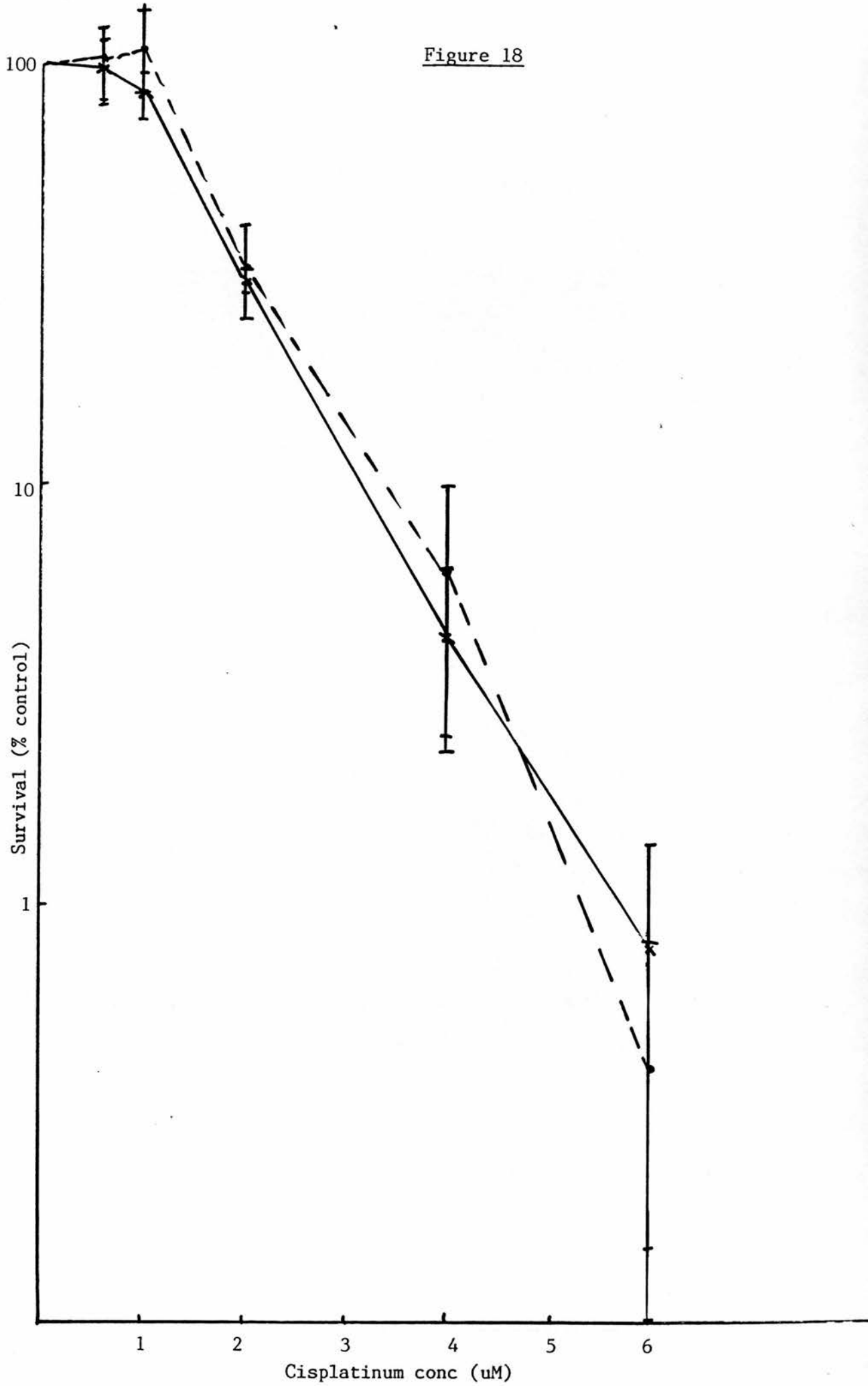


Figure 19 CisPlatinum Sensitivity - 1 hour dose

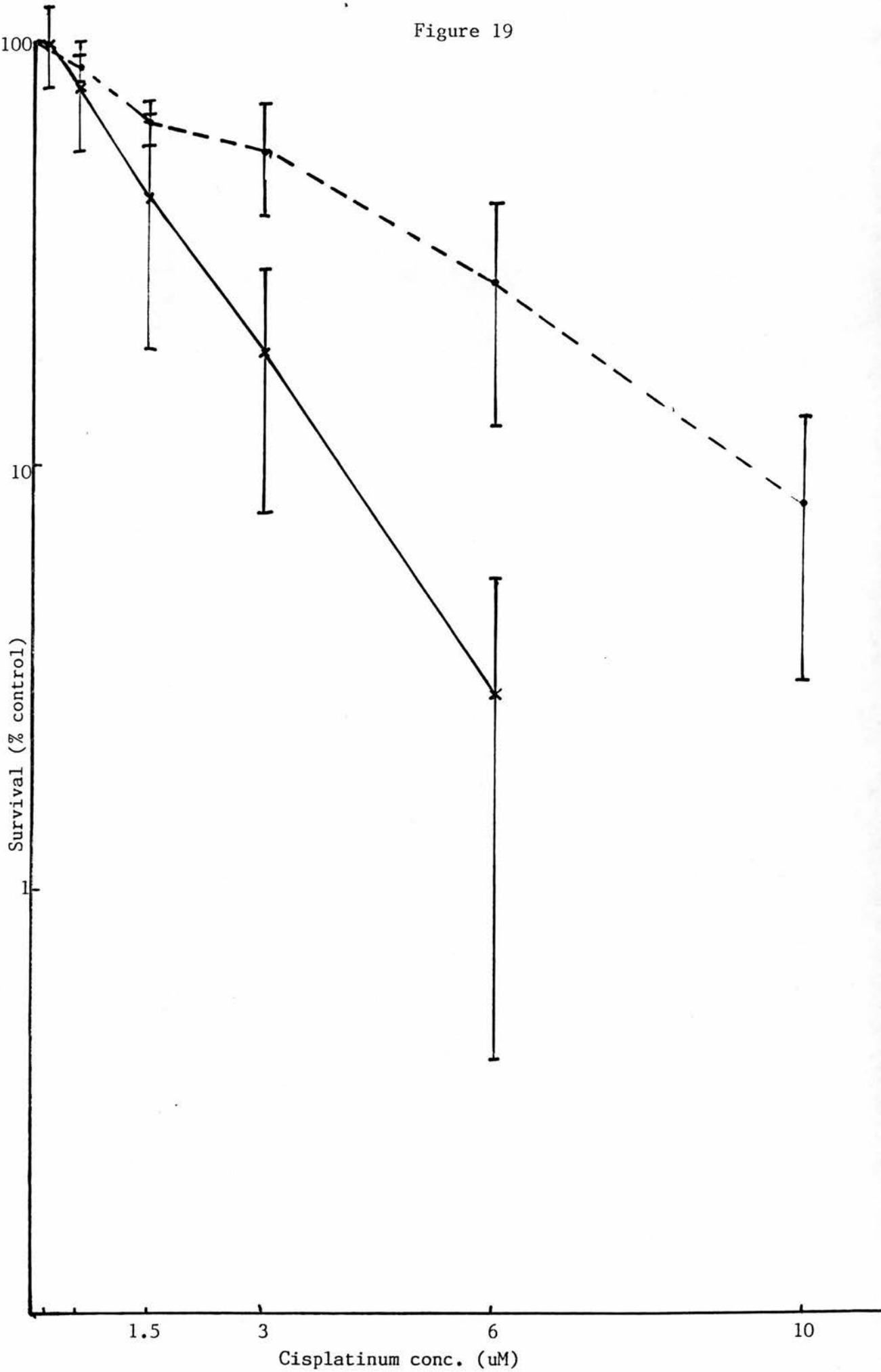
PE/01 ×——×

PE/04 ●- - - -●

Dose (uM)	% Survival[a] PE/01	PE/04
0.2	98.7 ± 21.5 (4) (99.4)	-
0.6	77.0 ± 21.9 (4) (76.3)	86.5 ± 6.3 (3) (98.4)
1.5	43.0 ± 24.0 (2) (35.8)	64.1 ± 7.9 (3) (62.5)
3.0	18.5 ± 10.8 (2) (22.0)	54.9 ± 16.2(2) (56.1)
6.0	2.9 ± 2.5 (2) (5.3)	27.0 ± 14.7(2) (24.0)
10.0	-	8.1 ± 5.0 (2) (10.3)

(a) notes as in figure 17

Figure 19



PE/01 and PE/04 remained the same. Some variation between experiments was observed but within any one experiment done at the same time with the same batch of drug the ratio of PE/04 to PE/01 sensitivity remained constant. This variation between experiments (as also noted by B.T. Hill et al, 1984) may have been due to the speed in which the drug diluted into medium was added to the cell cultures, particularly in a large experiment where 1 hour, 6 hour and 3 day exposures on both cell lines were attempted together. That experiment is shown in Figure 20 where the effect of increasing exposure time can be observed. For PE/01 the survivals were 119.6%, 47.6% and 7.5% for 1 hour, 6 hour and 3 day exposures of 0.6uM cisplatinum compared with 44.1%, 5.4% and 0.1% in a smaller experiment. The result for PE/04 at 1.5uM cisplatinum was 79.2%, 41.4%, 0.4% compared with 37.6%, 0.3% and 0% in a small experiment.

The possible effects of cisplatinum decomposition on the cell survival experiments have not been pursued further in this project and in all experiments cisplatinum solutions were made up from frozen stock solutions in saline at -20^{o}C and used immediately.

3.3.2 Chlorambucil

Courtenay assay

Combined results from 5 experiments with each cell line

Figure 20 CisPlatinum Sensitivity - Time schedule of dose

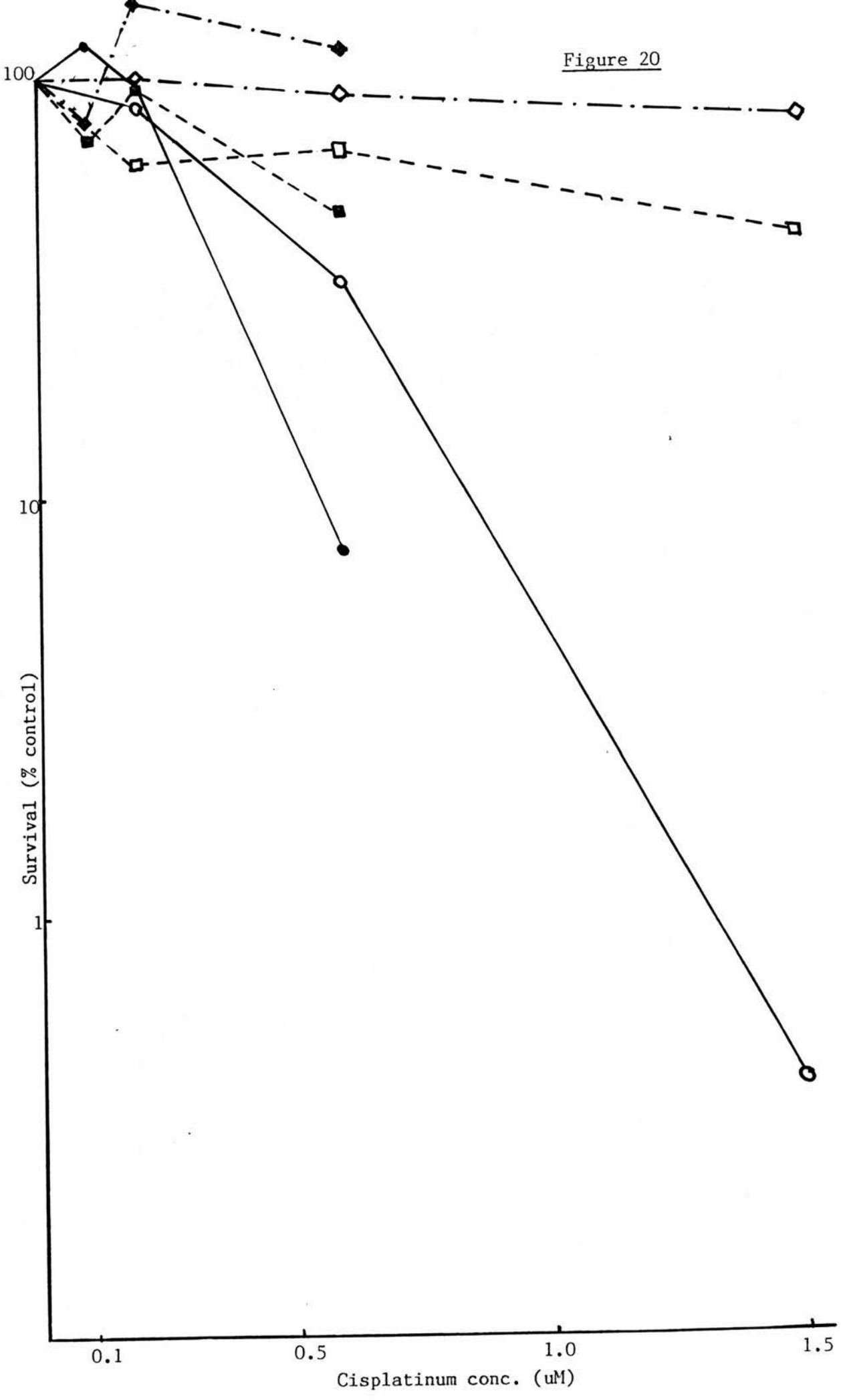
PE/01 ● 3 day dose, ■ 6 hour dose, ◆ 1 hour dose

PE/04 ○ 3 day dose, □ 6 hour dose, ◇ 1 hour dose

	Cisplatinum Dose (uM)					
	0.02	0.06	0.1	0.2	0.6	1.5
PE/01						
1 hour dose	125.4	112.4	78.6	151.5	119.6	-
6 hour dose	135.7	137.0	71.7	94.6	47.6	-
3 day dose	154.0	278.6?	120.4	96.3	7.5	-
PE/04						
1 hour dose	-	105.3	141.9	100.8	90.1	79.2
6 hour dose	-	90.5	70.7	62.5	67.7	41.4
3 day dose	-	88.9	136.1	84.9	32.6	0.4

(Survival was calculated by the [^{3}H]-thymidine pulse method. Visual colony counts for the highest 2 doses for each cell line were in good agreement.)

Figure 20



are shown in Figure 21. While in each experiment the dose response curve for survival of PE/04 was higher than PE/01 any difference can be seen to be minimal at approximately 1.3-fold. Four experiments on the 2 lines were done at the same time and a paired t-test on the slopes of the dose response lines showed no significant difference although on the LD_{50s} the difference was significant at the 5% level. The LD_{50s} were approximately 1.5uM and 2uM for PE/01 and PE/04 respectively.

Clonogenic assay on plastic

It was immediately apparent that PE/04 was more resistant to chlorambucil than PE/01 in this assay unlike the results in the Courtenay assay above. The combined results of 3 experiments for PE/01 and 4 experiments for PE/04 are shown in Figure 22. Also shown are the results from 7 experiments with the PE/01 $CisPt^R$ line. A 3-fold difference in sensitivity was observed with LD_{50s} of 0.83 and 2.8uM chlorambucil for PE/01 and PE/04 respectively. PE/01 $CisPt^R$ was only 4 times more resistant than its parent line, similar to the sensitivity of PE/04.

Later in this project when a 5% O_2 incubator was available, this assay was repeated in 5% O_2 to mimic the gas conditions in the Courtenay assay and a reduced difference between the 2 cell lines PE/01 and PE/04 was observed. Results from 1 experiment where duplicate

Figure 21 Chlorambucil Sensitivity - Courtenay assay

PE/01 ×——×

PE/04 ●- - -●

Dose (uM)	% Survival[a] PE/01	PE/04
0.1	44.4 ± 8.5 (1)[b]	112.9 ± 37.2 (2)
0.2	76.7 ± 14.1(2)	65.0 ± 0.3 (2)
0.5	49.2 ± 10.5(1)[b]	82.1 ± 16.9 (2)
0.7	67.6 ± 13.3(2)	70.4 ± 3.0 (2)
1.0	70.3 ± 12.2(3)	92.4 ± 8.6 (2)
2.0	37.5 ± 6.3 (3)	47.6 ± 6.4 (4)
5.0	10.7 ± 4.1 (5)	19.0 ± 5.5 (5)
8.0	0.2 ± 0.2 (1)	1.0 ± 0.5 (1)
20.0	0.08± 0.08(2)	1.1 ± 0.8 (1)

(a) notes as in figure 16

(b) from experiment with only 25 colonies in control tubes but showing a good dose response curve. Points not shown on the graph.

Figure 21

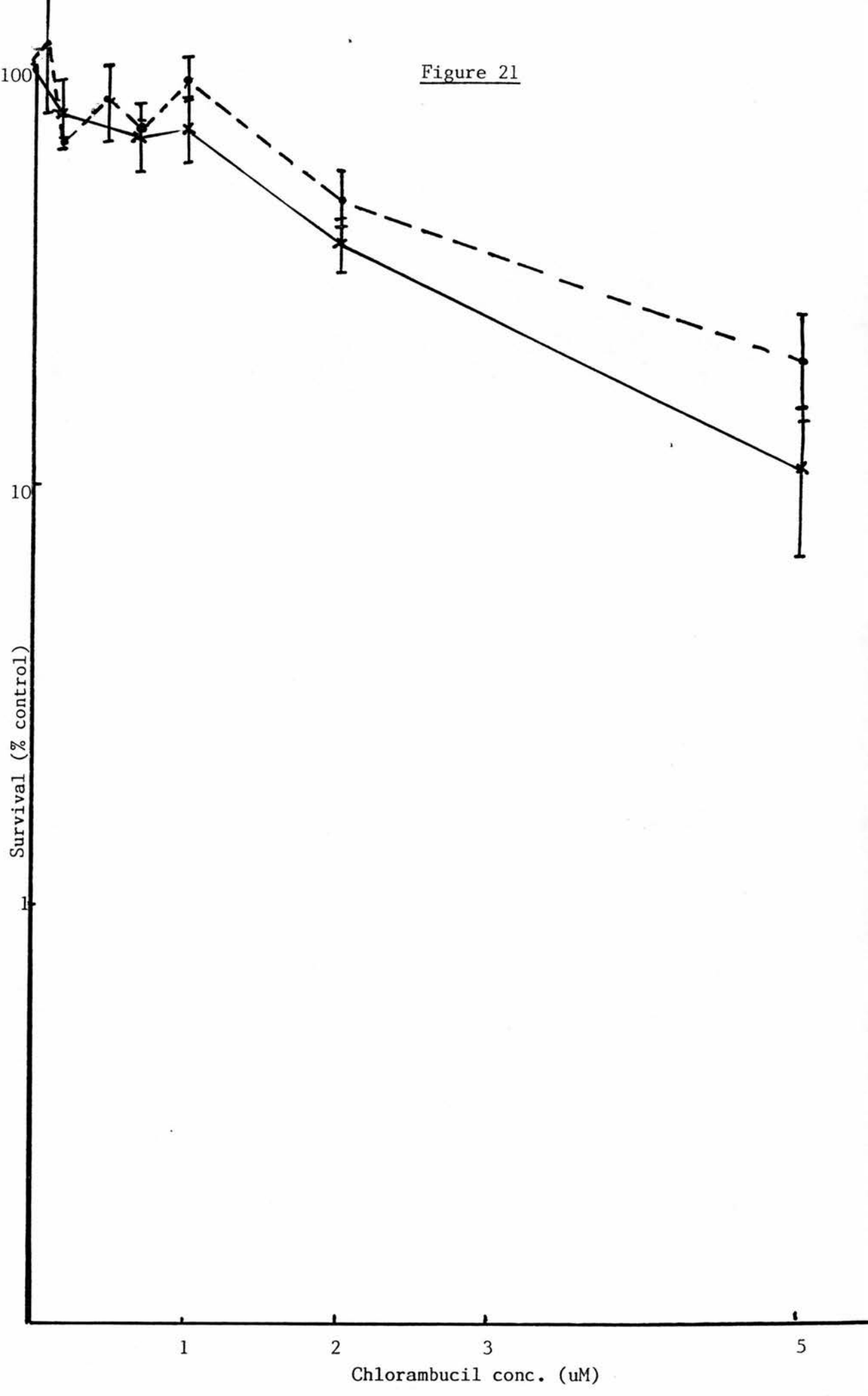


figure 22 Chlorambucil Sensitivity - Clonogenic assay on plastic

PE/01 ×——×

PE/04 ●- - - -●

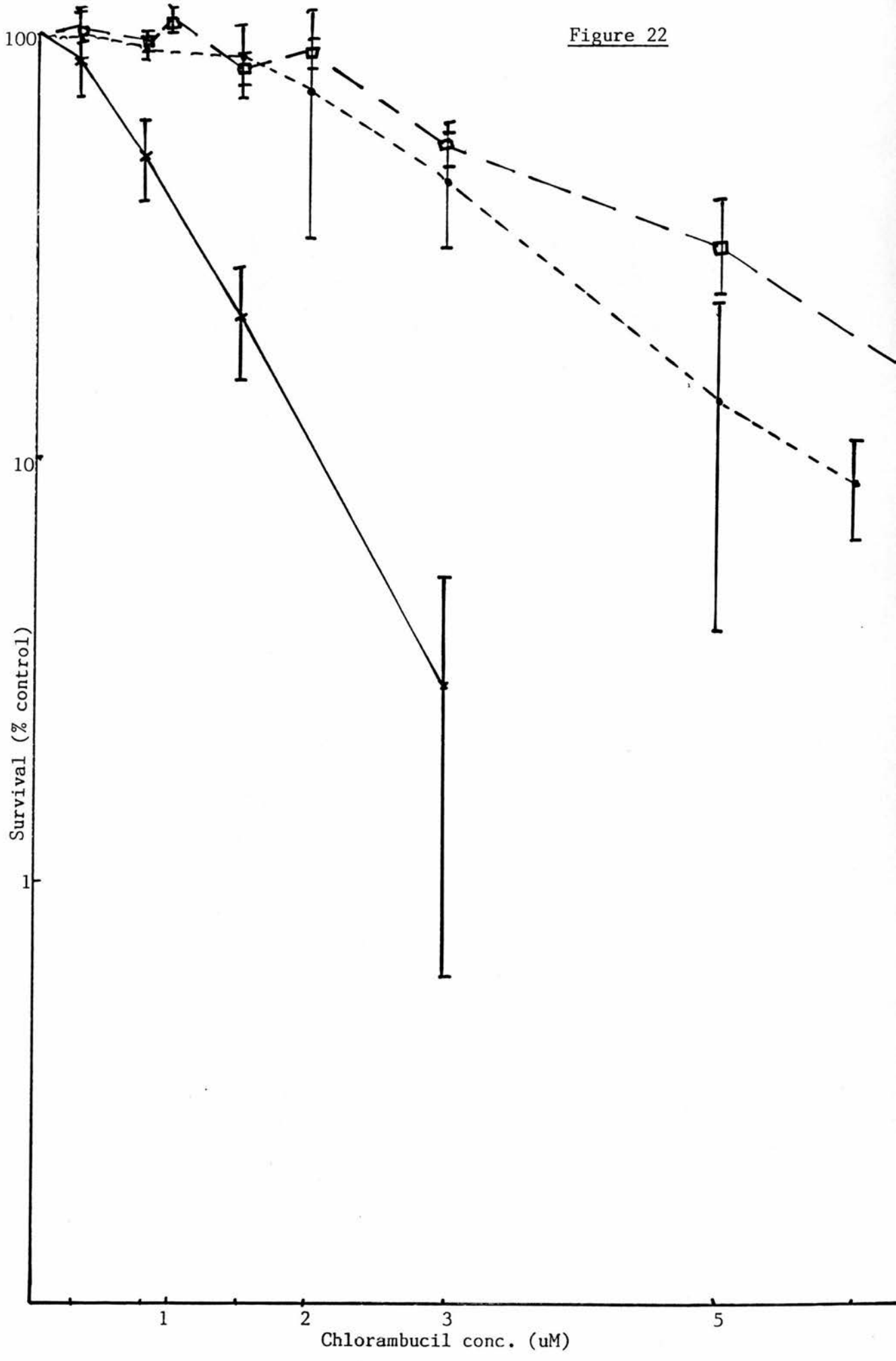
PE/01 CisPt^R □— — —□

Dose (uM)	% Survival PE/01(a)	PE/04(a)	PE/01 CisPt^R(b)
0.3	87.4 ± 16.1(3) (75.4)	99.5 ± 13.6(3) (111.6)	102.4 ± 14.5(2) (99.5)
0.4	-	87.9 (1)	-
0.8	51.9 ± 11.0(2) (38.1)	93.2 ± 5.5 (3) (93.7)	96.2 ± 4.5 (2) (99.9)
1.0	46.6 (1)	87.5 (1)	110.0 ± 6.2 (3) (107.4)
1.5	21.7 ± 6.4 (2) (16.1)	88.0 ± 16.3(2) (105.5)	83.1 ± 6.7 (2) (79.4)
2.0	15.6 (1)	73.8 ± 40.5(2)	90.2 ± 6.9 (4) (70.2)
3.0	2.9 ± 2.3 (2) (3.6)	45.5 ± 13.7(2) (50.8)	55.7 ± 6.6 (7) (51.6)
5.0	0.24 (1)	13.6 ± 9.7 (2)	32.9 ± 8.5 (4) (33.4)
6.0	0 (2) (0.96)	8.7 ± 2.3 (2) (9.7)	-
8.0	-	-	7.1 ± 2.3 (7) (8.4)
10.0	0 (1)	0.8 (1)	-
15.0	-	-	0 (1)

(a) results shown are those using the visual colony count since the [^{3}H]-thymidine pulse method was not used in some of the earlier experiments. The number of experiments is shown in brackets with the bracket underneath showing the result using the [^{3}H]-thymidine method.

(b) results using the visual colony count method are shown and graphed to compare with PE/01 and PE/04 although allwells of triplicates were not always counted. Results using the [^{3}H]-thymidine pulse method are shown underneath in brackets.

Figure 22



plates were incubated in 5%CO_2/air or 5%O_2/5%CO_2/90%N_2 are shown in Figure 23. The combined results of 2 experiments at 5%O_2 are shown in Figure 24. The difference between the 2 cell lines was only about 1.5 fold with LD_{50s} of 2.8uM and 4.3uM for PE/01 and PE/04 respectively.

3.3.3 5-fluorouracil

Courtenay assay

The results using 5-fluorouracil were quite variable between experiments with no cell survival at 0.1uM for both cell lines in one experiment and little toxicity up to 2uM in another. One experiment between these extremes where a dose response was obtained is shown in Figure 25. However in any one experiment when both cell lines were assayed at the same time no difference in sensitivity between them was observed (in 4 experiments). LD_{50s} were 1uM or lower in all but one experiment.

Clonogenic assay on plastic

Variability between experiments was again noticeable although not to such an extreme extent as that observed above. No significant difference between the two cell lines was noticed when they were tested together (4 experiments - if anything PE/04 was slightly more sensitive in 2 experiments and slightly less sensitive in 1 experiment). LD_{50} values were approximately 6uM for PE/01 and 4uM for PE/04 (taking the average from 4

Figure 23 Chlorambucil Sensitivity at 5% vs 20% Oxygen tension

PE/01 ● 20% O_2, ■ 5% O_2 ——

PE/04 ○ 20% O_2, □ 5% O_2 - - - - -

Results from 1 experiment. Each point is the mean of triplicates.
Standard error bars omitted for clarity.

Figure 23

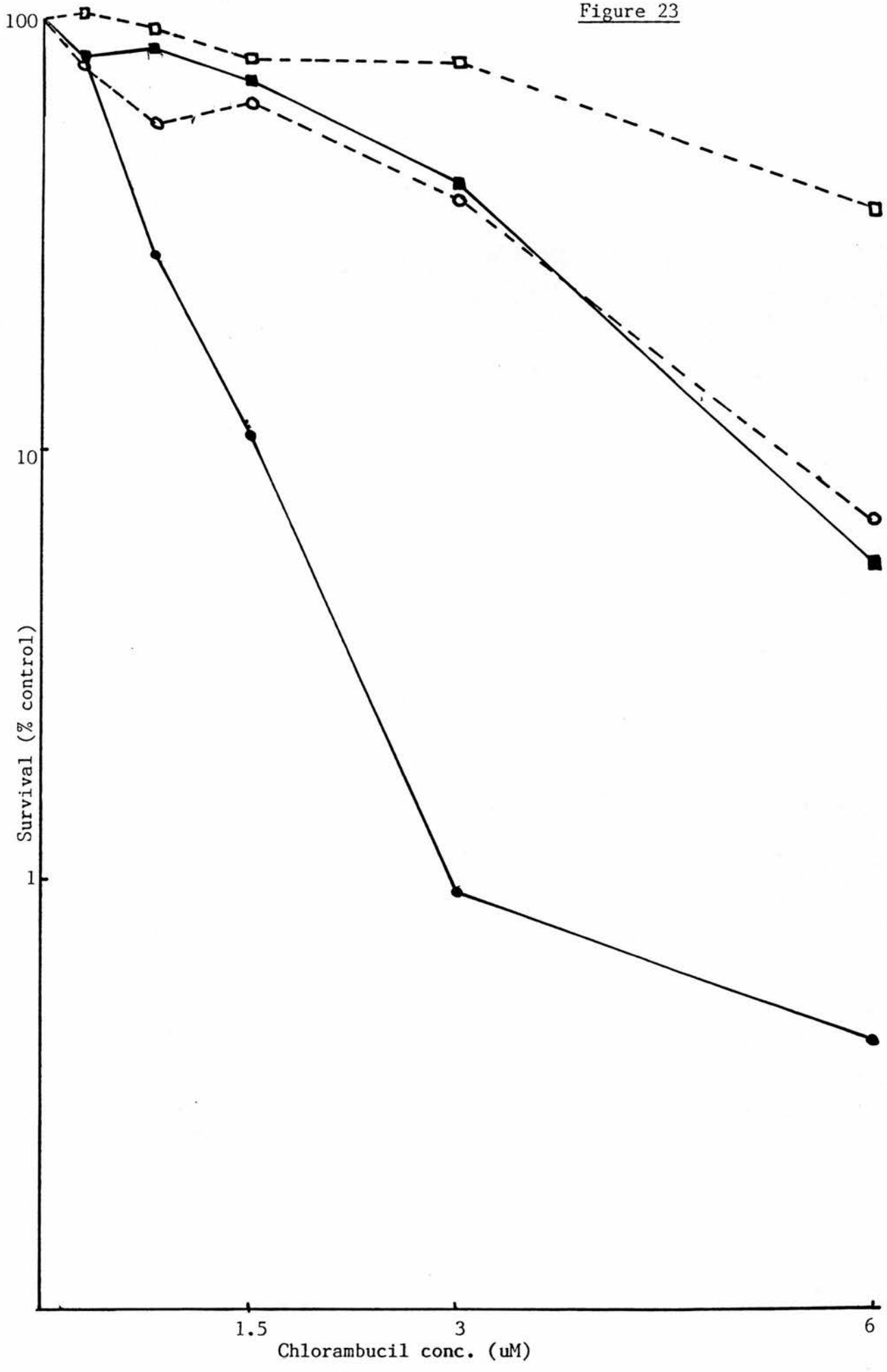


Figure 24 Chlorambucil Sensitivity - at 5% Oxygen tension

PE/01 ✕——✕

PE/04 ●- - -●

Dose (uM)	% Survival[a] PE/01	PE/04
0.3	93.6 ± 12.4 (89.4)	99.7 ± 3.7 (99.9)
0.8	86.5 ± 1.8 (82.4)	90.9 ± 3.6 (86.1)
1.5	81.7 ± 9.7 (74.3)	82.0 ± 1.3 63.7)
3.0	46.8 ± 5.4 (44.8)	69.8 ± 9.1 (56.9)
6.0	7.5 ± 2.2 (5.6)	33.9 ± 2.1 (28.9)

(a) results combined from 2 experiments.
notes as in figure 17.

Figure 24

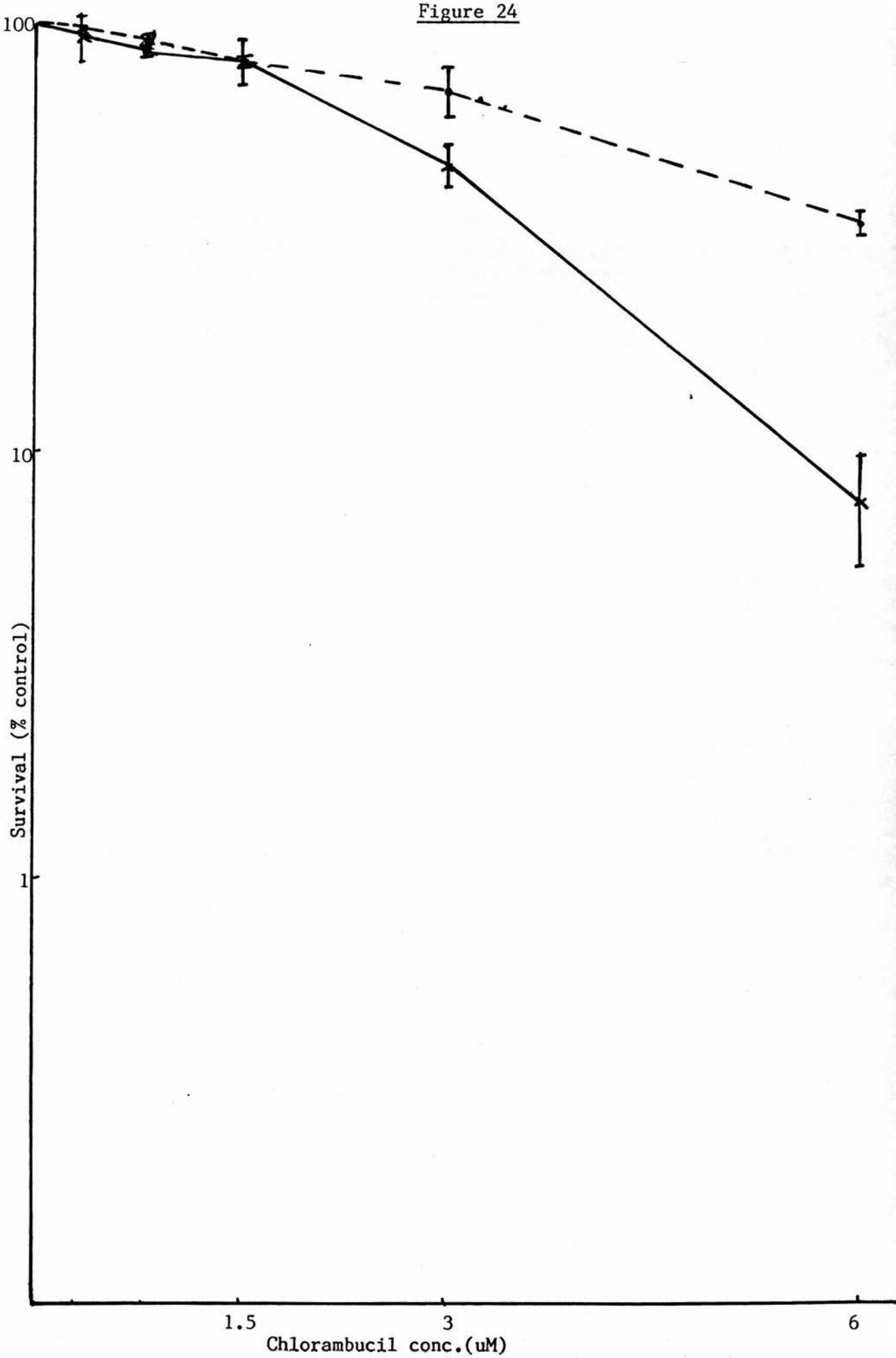
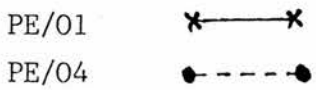


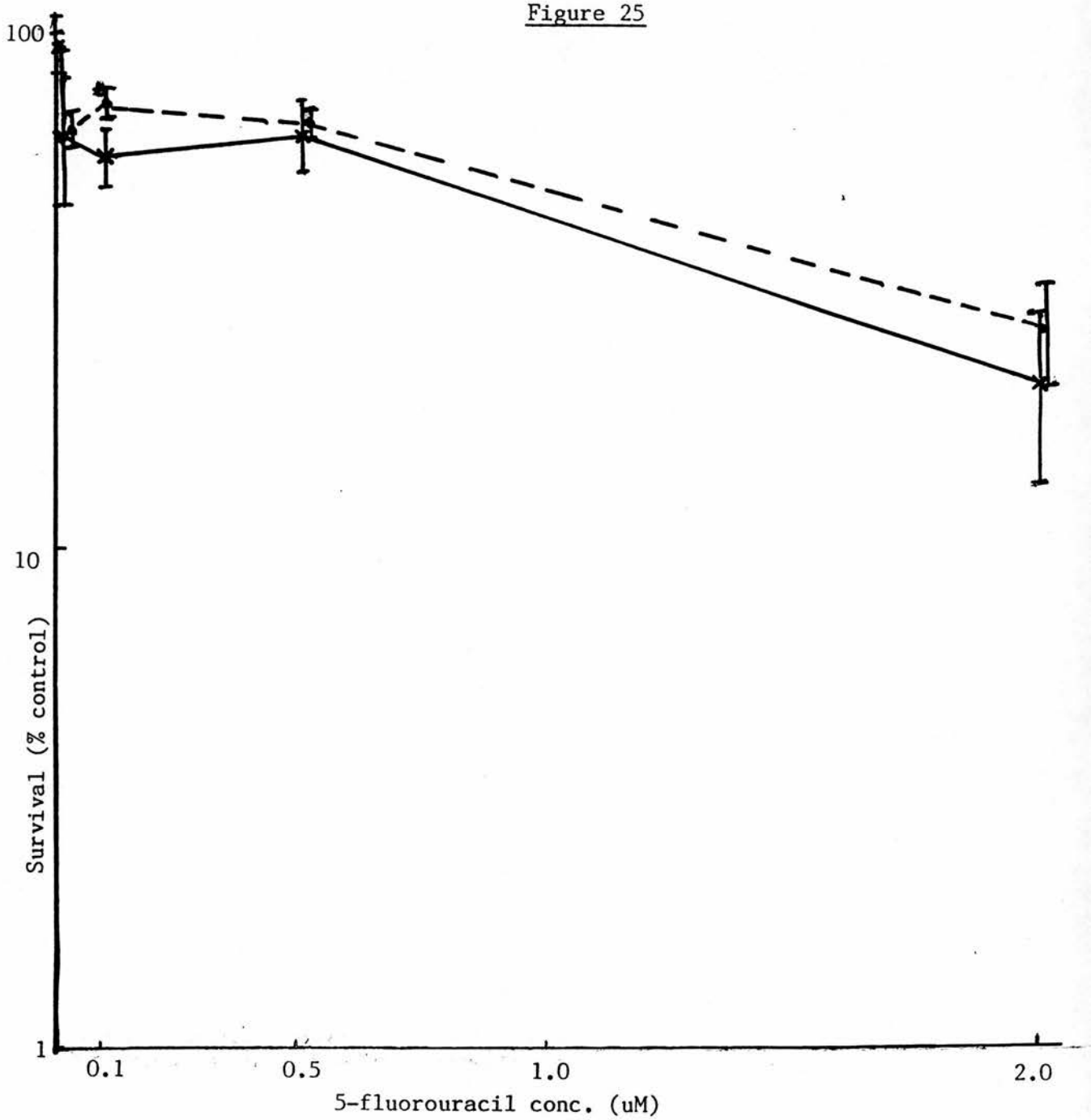
Figure 25 5-fluorouracil Sensitivity - Courtenay assay



5-fluorouracil dose left on for duration of the assay.

The results of one experiment are shown since variations between experiments were observed. Mean and standard error of 5 replicates are shown.

Figure 25



experiments for PE/01 and 5 experiments for PE/04). The LD_{50} figure in different experiments varied between 2uM and 12uM and can also vary depending on the method of estimation. One fairly representative experiment is shown in Figure 26.

3.3.4 Drug sensitivity of early passage PE/01 and PE/04 cultures

As shown above (Figure 16) no variation in sensitivity to cisplatinum with passage in culture was observed for PE/04 with the Courtenay assay. Recently this assay has been further used to check sensitivity at low passage numbers in PE/01 and PE/04 to all 3 of the clinically used drugs. 1-3 x 10^4 and 5 x 10^3 cells were needed for PE/01 and PE/04 respectively due to the lower colony forming efficiency at early passages. Essentially the same results as at higher passages have been obtained at passages 6 and 12 for PE/01 and passages 2 and 11 for PE/04 in initial experiments and this work is continuing in this laboratory. One initial experiment with cisplatinum has also been attempted so far with the PE/04 ascites previously frozen in liquid nitrogen with comparable results to the cell line.

3.4 Cross-resistance to other drugs (PE/01, PE/04 and PE/01 $CisPt^R$ lines)

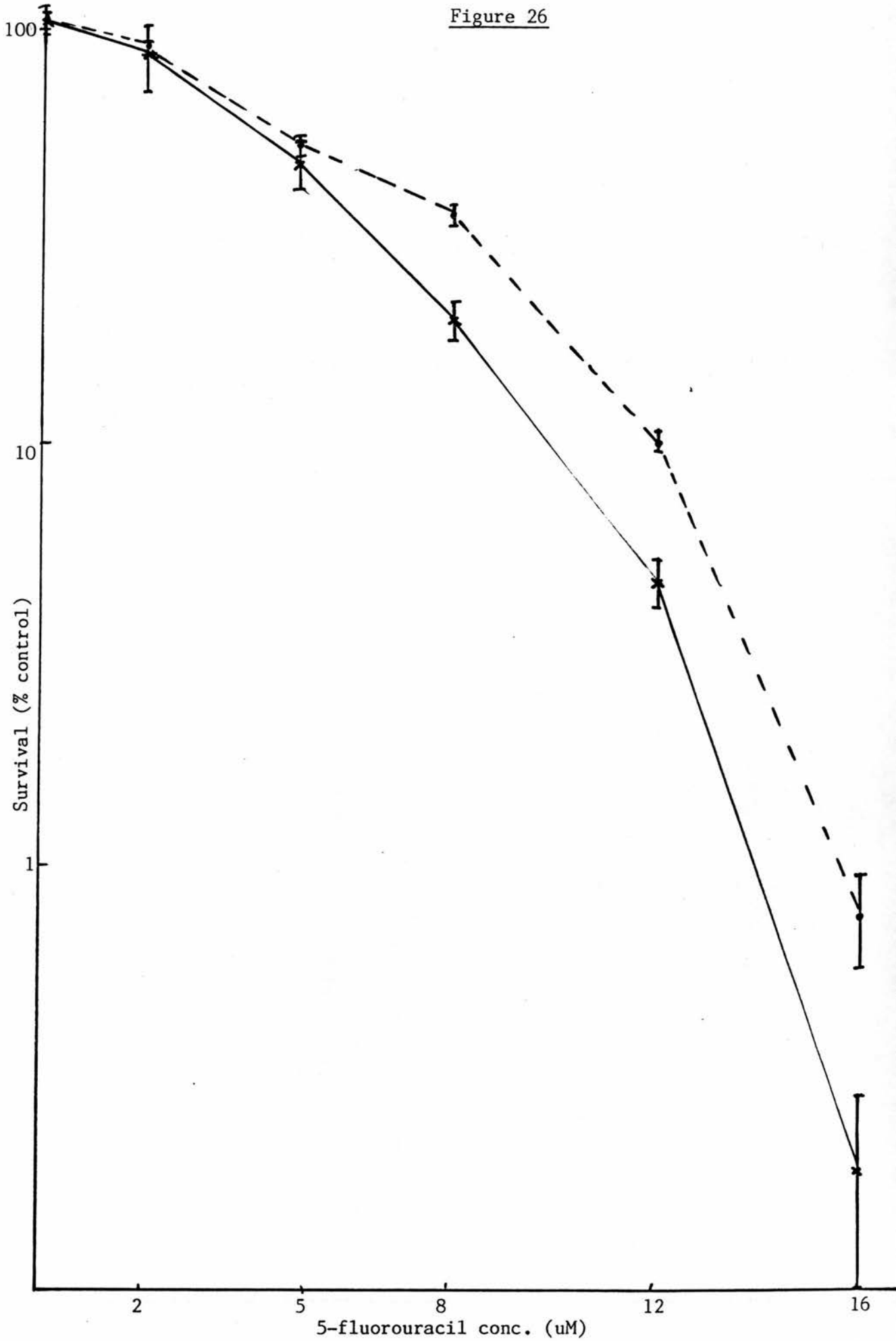
Other drugs were tested in the cell lines PE/01, PE/04 and

Figure 26 5-fluorouracil Sensitivity - Clonogenic assay on plastic

(dose of 5-fluorouracil left on for 3 days)

The results of one experiment are shown since variations between experiments were observed. Mean and standard of triplicates in this experiment are shown.

Figure 26



PE/01 CisPt^R to see what patterns of cross-resistance or collateral sensitivity might be present. The relative sensitivities of the lines could give important clues to what mechanisms of resistance might be operating. The platinum analogues CBDCA, CHIP and JM-40 were tested to compare with cisplatinum. Another classical alkylating agent, melphalan, was tested to compare with chlorambucil and also with cisplatinum since resistance to melphalan and cisplatinum in vitro has often been associated (T.C. Hamilton et al, 1985). Doxorubicin and vincristine were tested as representatives of other major classes of antineoplastic drugs and the sensitivity to x-irradiation was also tested. The newer drugs mitozantrone, an anthracene dione, and prednimustine, a modified alkylating agent composed of chlorambucil and prednisolone moieties, were tested since others in the Department were interested in the activity of these new clinical drugs. The clonogenic assay on plastic was used for all these experiments.

The results for the various drugs are shown in Figures 27-35 and a summary of LD_{50} values is shown in Table 10 together with the relative fold resistance compared with PE/01. While the LD_{50} values are a concise way of looking at differences the dose response curves give a better idea as to the significance of these differences across the dose range. Differences in the LD_{50} value can be due to an initial shoulder on the curve, a change in the slope or

Figure 27 CHIP Sensitivity

PE/01 ×——×

PE/04 ●- - -●

PE/01 CisPt^R □— — —□

(3 day exposure in clonogenic assay on plastic)

Dose (uM)	% Survival(a) PE/01	PE/04	PE/01 CisPt^R
0.06	70.8 (1) (102.7)	77.2 (1) (108.8)	-
0.1	79.0 (1) (97.8)	86.0 (1) (75.6)	-
0.2	66.0 ± 5.0 (3) (68.1)	95.0 ± 14.0(5) (86.9)	-
0.5	46.7 ± 16.4(2) (43.0)	72.5 ± 15.7(3) (60.8)	-
0.6	23.1 (1) (30.7)	37.6 ± 1.8 (2) (35.8)	112.9 (1) (95.2)
0.7	46.8 ± 10.1(2) (48.3)	47.3 ± 11.3(3) (43.9)	-
1.0	32.8 ± 16.1(2) (23.1)	29.8 ± 4.5 (3) (27.0)	98.9 ± 15.5(4) (91.6)
1.5	-	2.6 ± 0.7 (2) (3.3)	-
2.0	8.5 ± 0.2 (2) (3.0)	2.2 ± 0.7 (3) (3.7)	100.3 (1) (81.3)
2.5	-	-	53.7 ± 2.1 (3) (61.3)
4.0	-	-	72.9 (1) (49.8)
5.0	-	-	16.1 ± 3.7 (3) (15.7)
6.0	-	-	40.6 (1) (10.1)
7.5	-	-	3.0 ± 1.0 (3) (2.2)
10.0	-	-	0.38± 0.08(2) (0.07)

(a) notes as in figure 17.
Only points with results from more than one experiment are shown on the graph.

Figure 27

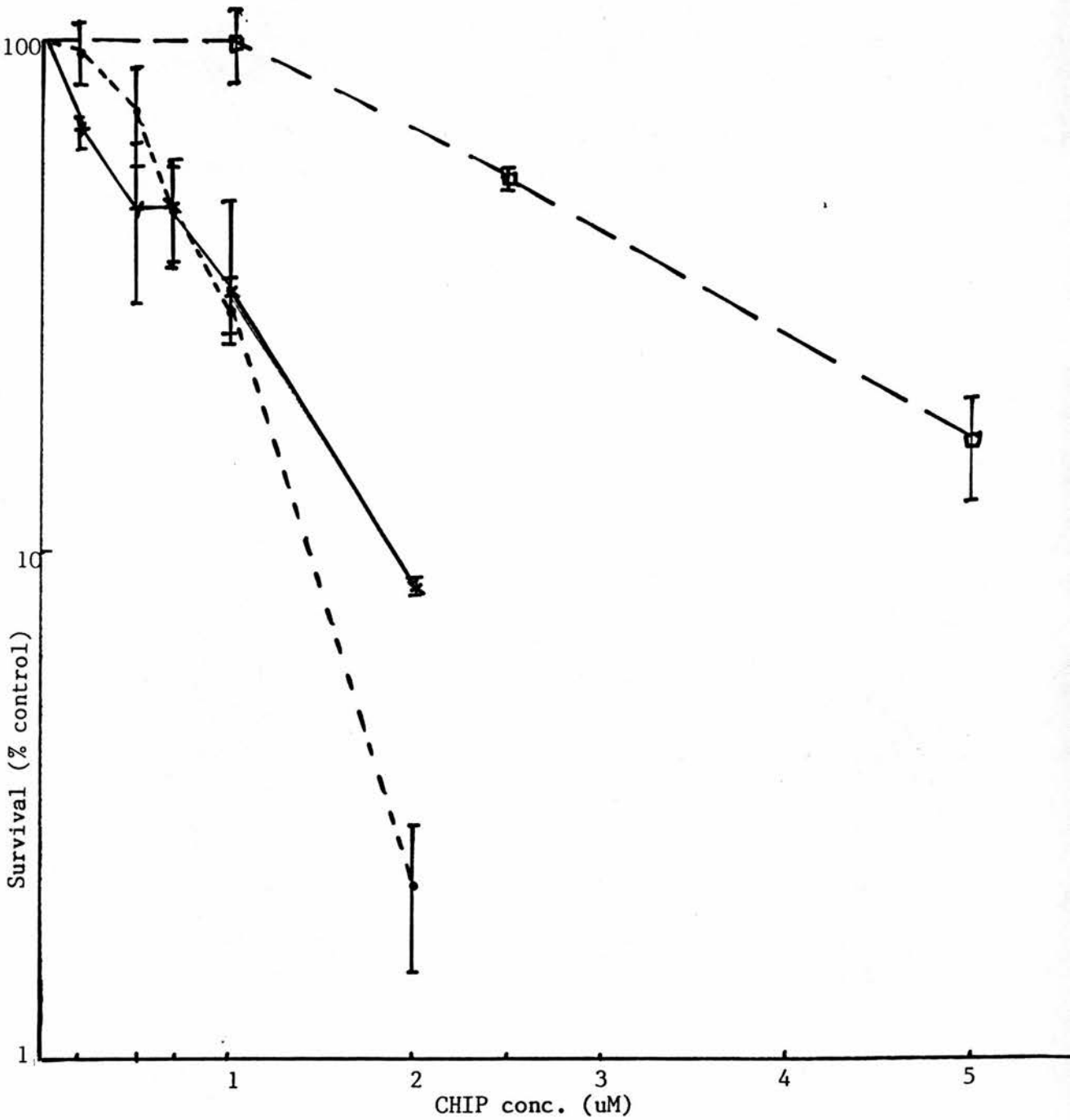


Figure 28 CBDCA Sensitivity

PE/01 ×——×

PE/04 ●- - - -●

PE/01 CisPt^R □— — —□

Dose (uM)	% Survival PE/01	PE/04	PE/01 CisPt^R
0.1	87.7 (1) (106.0)	130.6 (1) (91.2)	-
0.5	93.0 ± 8.0 (3) (73.6)	82.2 ± 7.6 (3) (81.8)	-
1.0	51.7 ± 20.0(3) (30.3)	93.5 ± 7.7 (3) (67.0)	110.5 ± 11.2(2)
2.0	12.4 ± 4.0 (3) (12.9)	54.1 ± 11.1(2) (46.3)	94.1 ± 9.8 (2) (116.0)
3.0	6.9 ± 5.8 (3) (2.5)	29.5 ± 9.9 (2) (19.2)	-
4.0	-	-	103.0 ± 15.6(5) (118.1)
5.0	0.5 ± 0.2 (4) (0.8)	2.9 ± 0.9 (3) (5.3)	73.9 ± 4.5 (3) (59.0)
6.0	-	-	64.5 ± 6.6 (3) (81.3)
7.5	-	-	46.9 ± 0.6 (2) (36.0)
10.0	0 (1) (0)	0 (1) (0)	33.5 ± 6.0 (3) (42.3)
15.0	-	-	9.3 ± 3.5 (3) (5.2)
20.0	-	-	2.0 ± 1.1 (3) (1.4)
25.0	-	-	0.4 (1) (0)

Notes as in figure 17.

Figure 28

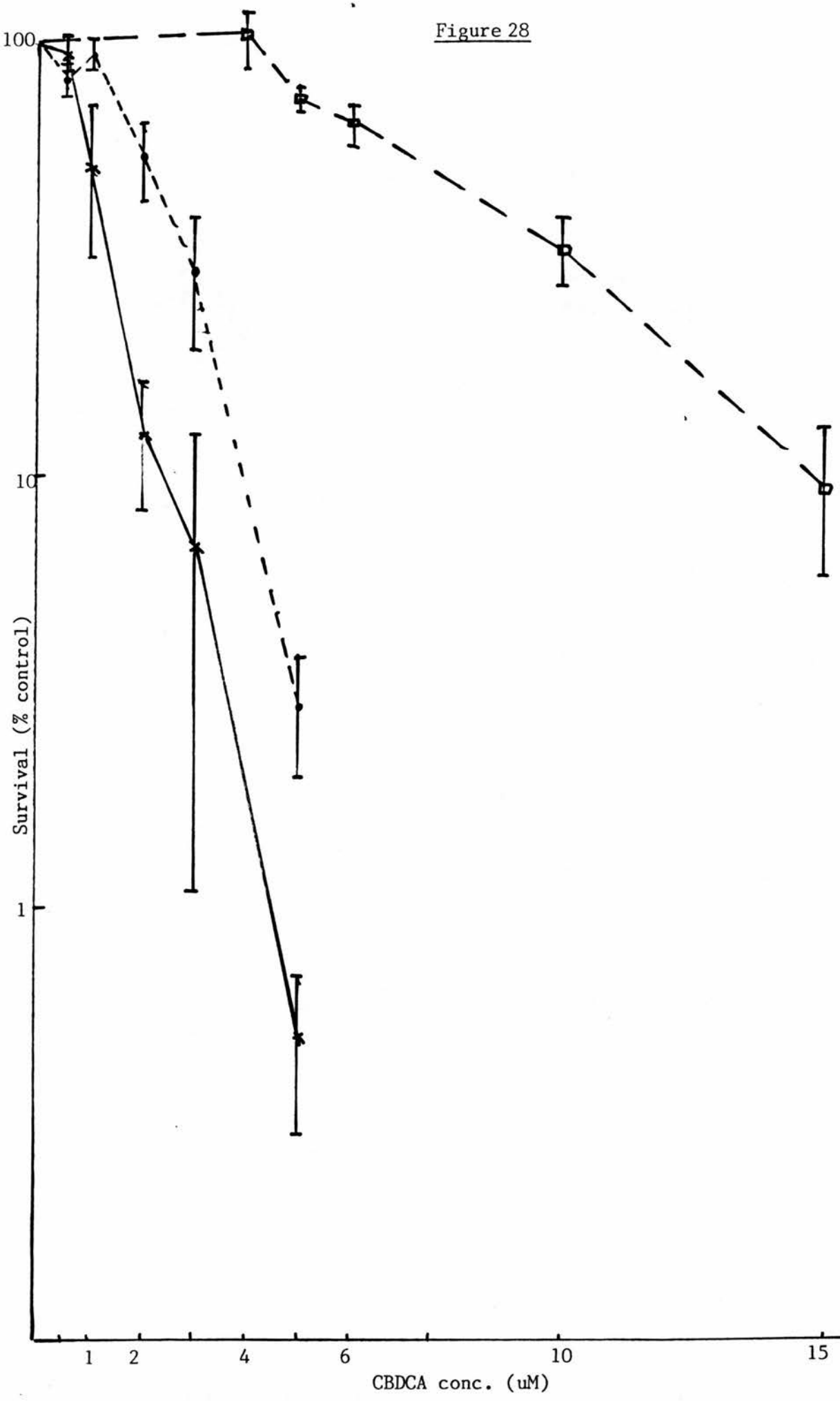
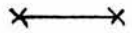


Figure 29 JM40 Sensitivity

PE/01 

PE/04 

PE/01 CisPt^R 

Dose (uM)	% Survival PE/01	PE/04	PE/01 CisPt^R
0.1	60.0 ± 4.2(4) (88.8)	92.8 ± 12.2(2) (96.8)	-
0.2	51.3 ± 2.9(2) (79.2)	93.8 ± 8.6 (2) (84.8)	-
0.5	33.9 ± 9.8(4) (46.9)	88.8 ± 19.0(4) (79.6)	-
1.0	20.2 ± 8.4(4) (30.5)	68.0 ± 8.6 (4) (72.2)	110.0 ± 11.1(2) (93.6)
2.0	8.9 ± 2.8(2) (20.9)	48.1 ± 4.2 (2) (50.7)	106.7 ± 11.2(2) (90.3)
4.0	-	-	71.3 ± 15.3(2) (71.9)
5.0	1.0 ± 0.8(2) (0.7)	11.4 ± 1.7 (4) (10.8)	75.3 ± 14.6(2) (78.7)
6.0	-	-	79.1 ± 2.8 (3) (59.1)
7.5	-	-	60.1 ± 3.2 (3) (65.1)
9.0	-	-	38.3 (1) (33.8)
10.0	0 (1) (0)	1.1 (1) (0.35)	43.7 ± 5.8 (3) (42.8)
15.0	-	-	12.1 (1) (7.9)
25.0	-	-	2.9 ± 1.1 (3) (2.0)
50.0	-	-	0.14± 0.14(3) (0)

Notes as in figure 17.

Figure 29

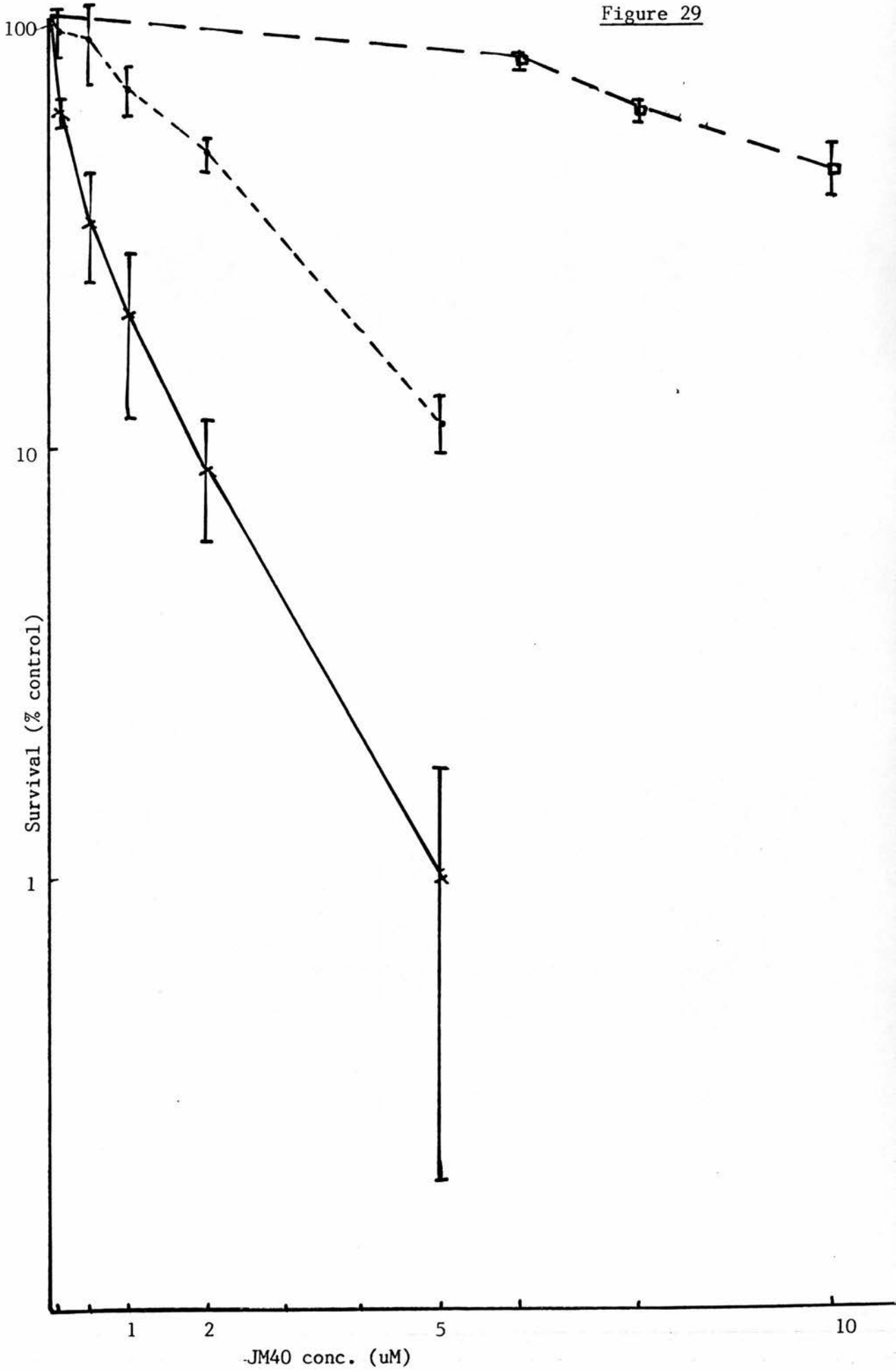


Figure 30 Melphalan Sensitivity

PE/01 ×——×

PE/04 ●- - - -●

PE/01 CisPt^R □—— —□

Dose (uM)	% Survival PE/01	PE/04	PE/01 CisPt^R
0.1	87.9 ± 47.0(2) (85.4)	-	-
0.3	49.9 ± 22.5(3) (58.4)	-	-
0.6	26.4 ± 13.3(3) (26.4)	81.6 ± 16.4(3) (90.9)	93.6 (1) (123.6)
0.8	29.0 ± 15.7(3) (22.4)	85.7 ± 16.9(3) (79.0)	72.4 ± 9.1(4) (100.8)
1.0	11.5 ± 3.9 (3) (8.7)	71.2 ± 14.8(3) (59.9)	59.0 ± 2.0(4) (68.8)
2.0	0.5 (1) (0)	26.8 ± 8.1 (3) (28.8)	58.3 ± 5.5(4) (73.5)
4.0	-	7.8 ± 4.9 (3) (7.3)	20.7 ± 1.9(4) (29.3)
6.0	-	-	7.0 ± 1.7(3) (9.0)

Notes as in figure 17.

Figure 30

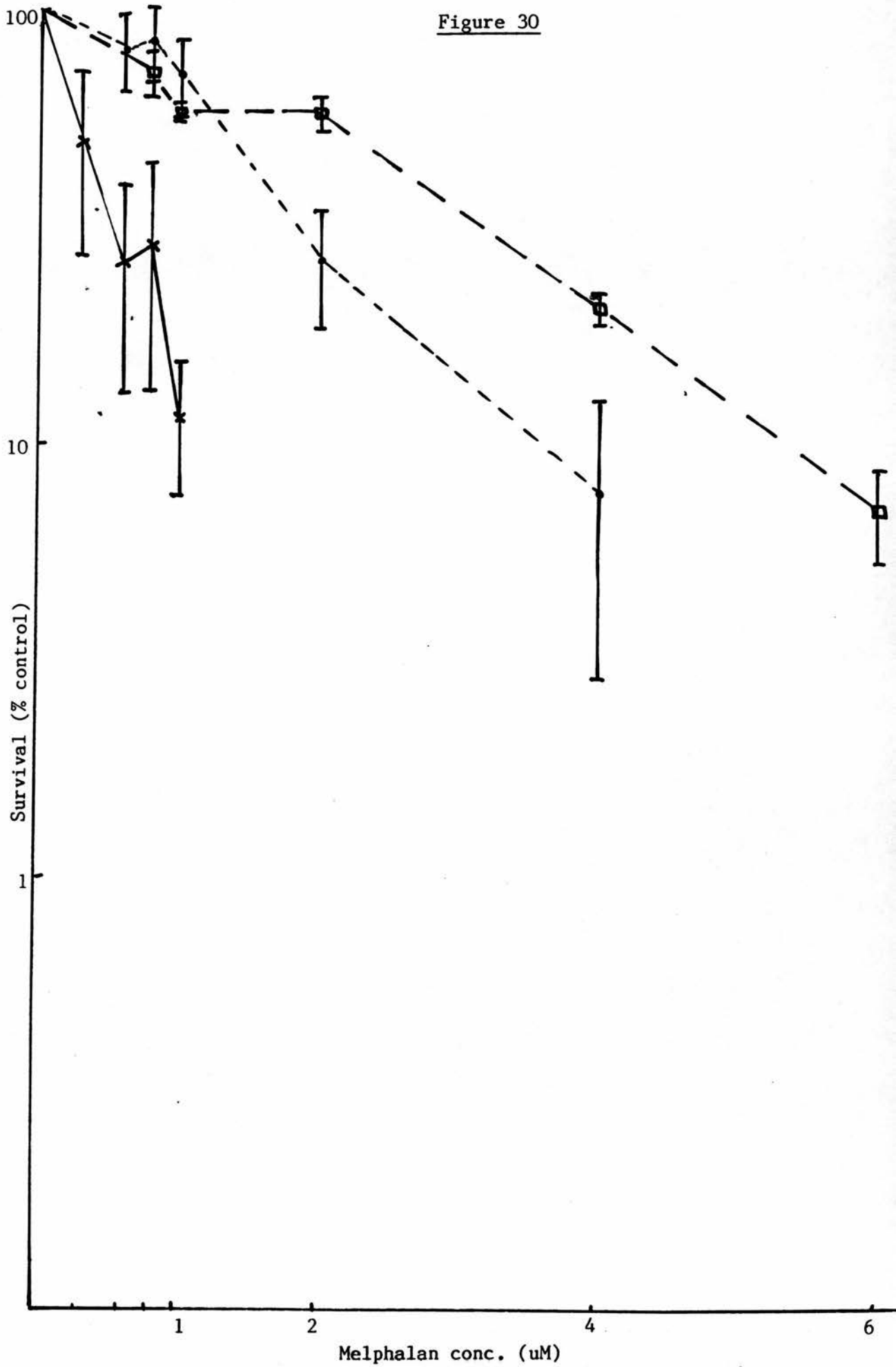


Figure 31 Doxorubicin Sensitivity

PE/01 ×——×

PE/04 ●- - - -●

PE/01 CisPt^R □— — —□

Dose (nM)	% Survival PE/01	PE/04	PE/01 CisPt^R
5.0	79.8 ± 13.6(3) (88.7)	82.4 ± 24.1(3) (79.7)	-
6.0	-	-	136.2 ± 1.5 (2) (86.8)
7.5	32.8 ± 10.1(2) (49.0)	85.3 ± 33.7(2) (71.5)	-
10.0	36.4 ± 13.5(3) (37.6)	76.5 ± 21.1(3) (90.4)	62.7 ± 15.7(5) (67.5)
15.0	-	-	40.0 ± 14.8(4) (59.1)
20.0	19.1 (1) (4.7)	33.2 (1) (42.1)	19.5 ± 4.0 (4) (35.8)
25.0	1.9 ± 0.6 (2) (2.8)	35.5 ± 8.2 (2) (31.4)	-
30.0	-	-	10.8 ± 5.3 (5) (13.0)
40.0	-	-	1.7 ± 0.8 (3) (2.4)
50.0	0 (2) (0)	5.7 ± 1.7 (3) (5.2)	-
60.0	-	-	0.9 ± 0.9 (2) (0)

Notes as in figure 17.

Figure 31

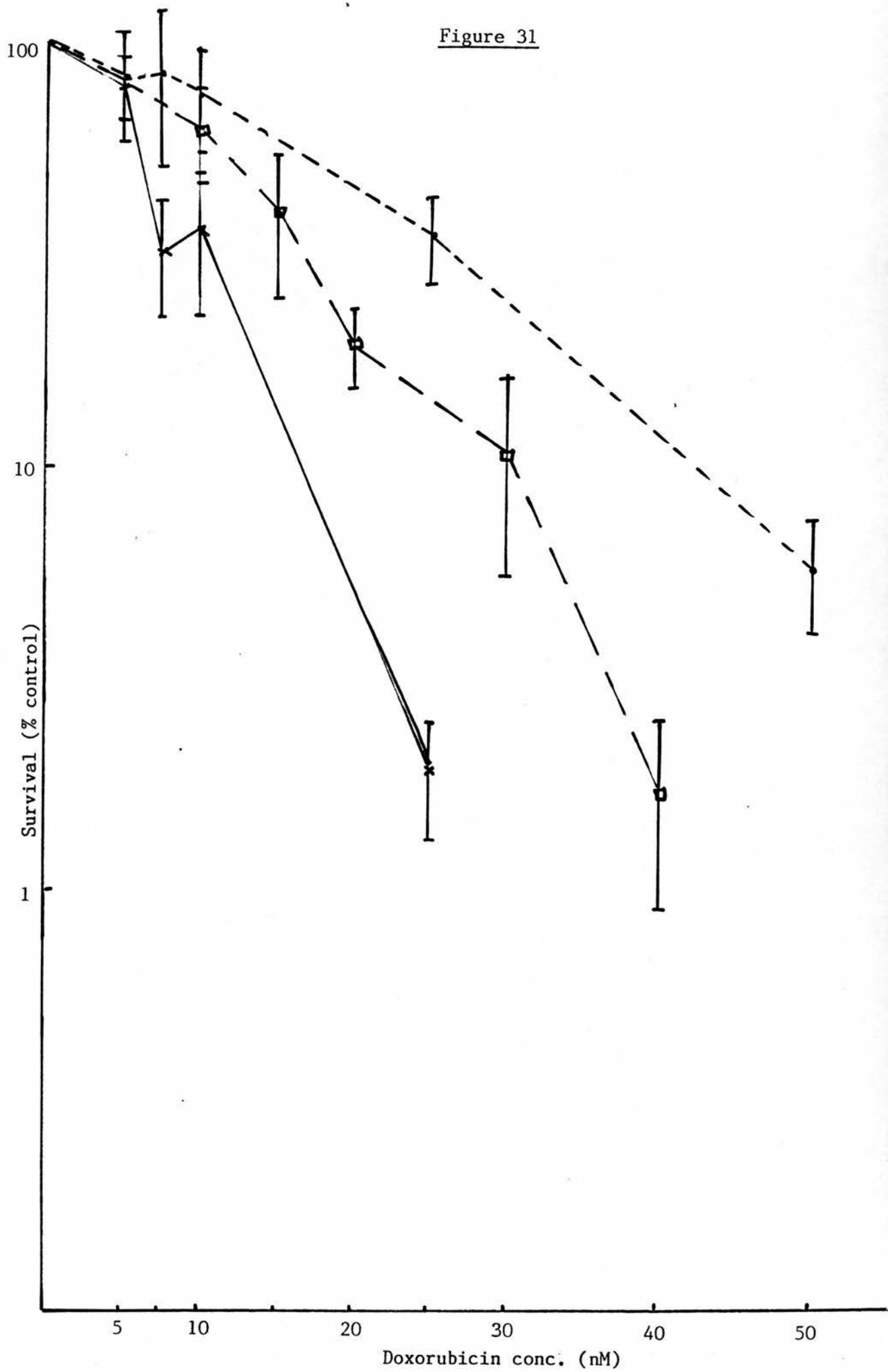


Figure 32 Vincristine Sensitivity

PE/01 ×——×

PE/04 ●- - -●

Dose (nM)	% Survival PE/01	PE/04
1.0	86.4 ± 13.1(3) (88.8)	90.6 ± 10.8(5) (100.6)
2.0	104.6 ± 29.9(3) (81.3)	98.7 ± 19.3(5) (99.7)
5.0	23.9 ± 10.2(3) (27.3)	57.0 ± 13.3(5) (39.1)
7.0	8.5 ± 4.9 (3) (6.6)	36.3 ± 11.5(5) (20.7)
10.0	3.0 ± 1.2 (3) (1.4)	9.6 ± 2.6 (5) (4.9)

Notes as in figure 17.

Figure 32

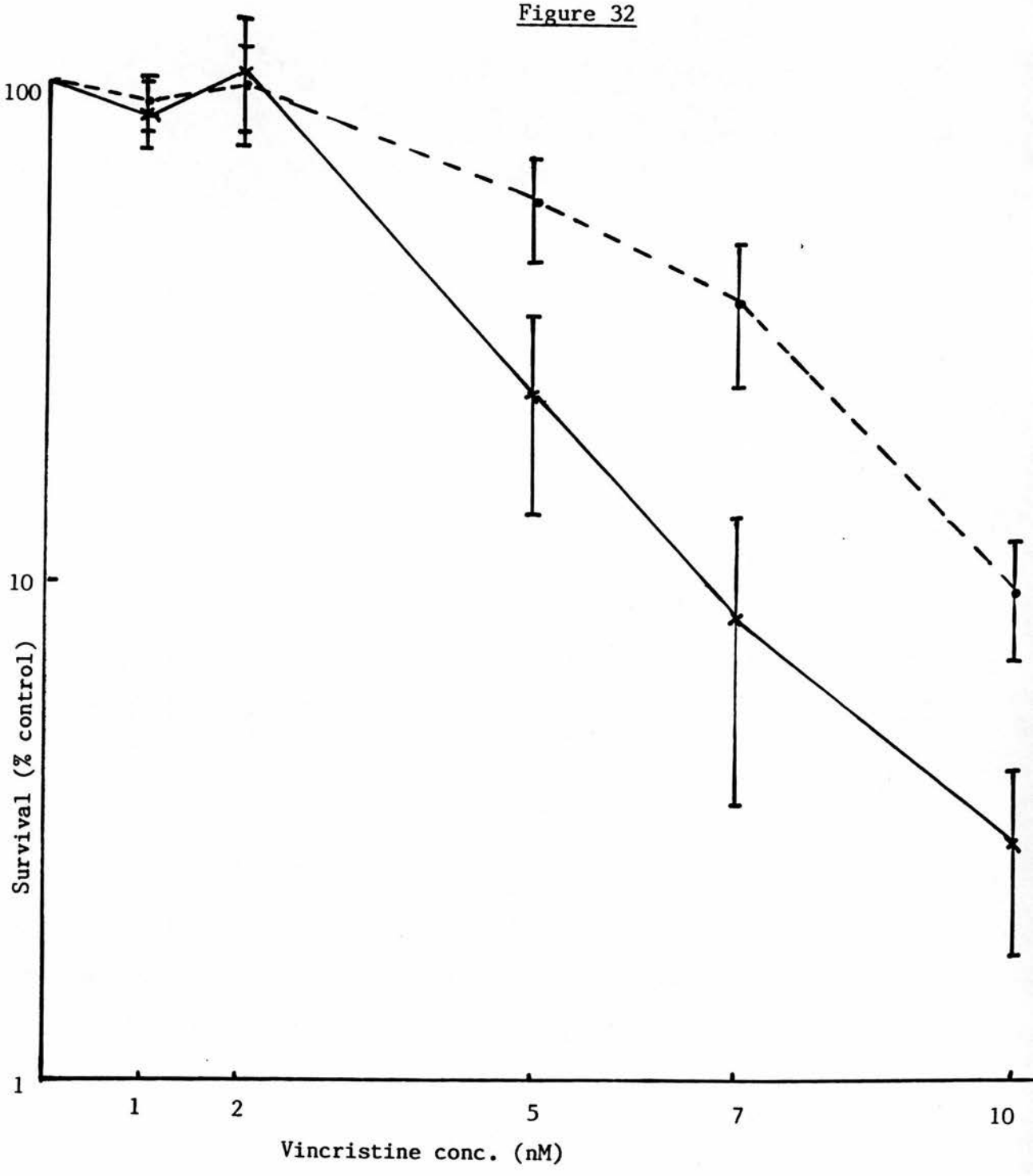


Figure 33 Mitozantrone Sensitivity

PE/01 ×——×

PE/04 ●- - -●

Dose (nM)	% Survival PE/01	PE/04
0.50	67.4 ± 6.6 (3) (80.5)	104.2 ± 9.1 (3) (89.2)
0.75	69.5 ± 20.8(2) (62.5)	87.0 ± 7.4 (2) (75.2)
1.0	47.2 ± 2.7 (3) (56.4)	68.5 ± 8.0 (3) (62.3)
2.5	14.7 ± 5.5 (2) (17.2)	45.3 ± 11.1(2) (28.1)
5.0	2.5 ± 0.2 (3) (0.73)	6.0 ± 0.3 (3) (1.5)

Notes as in figure 17.

Figure 33

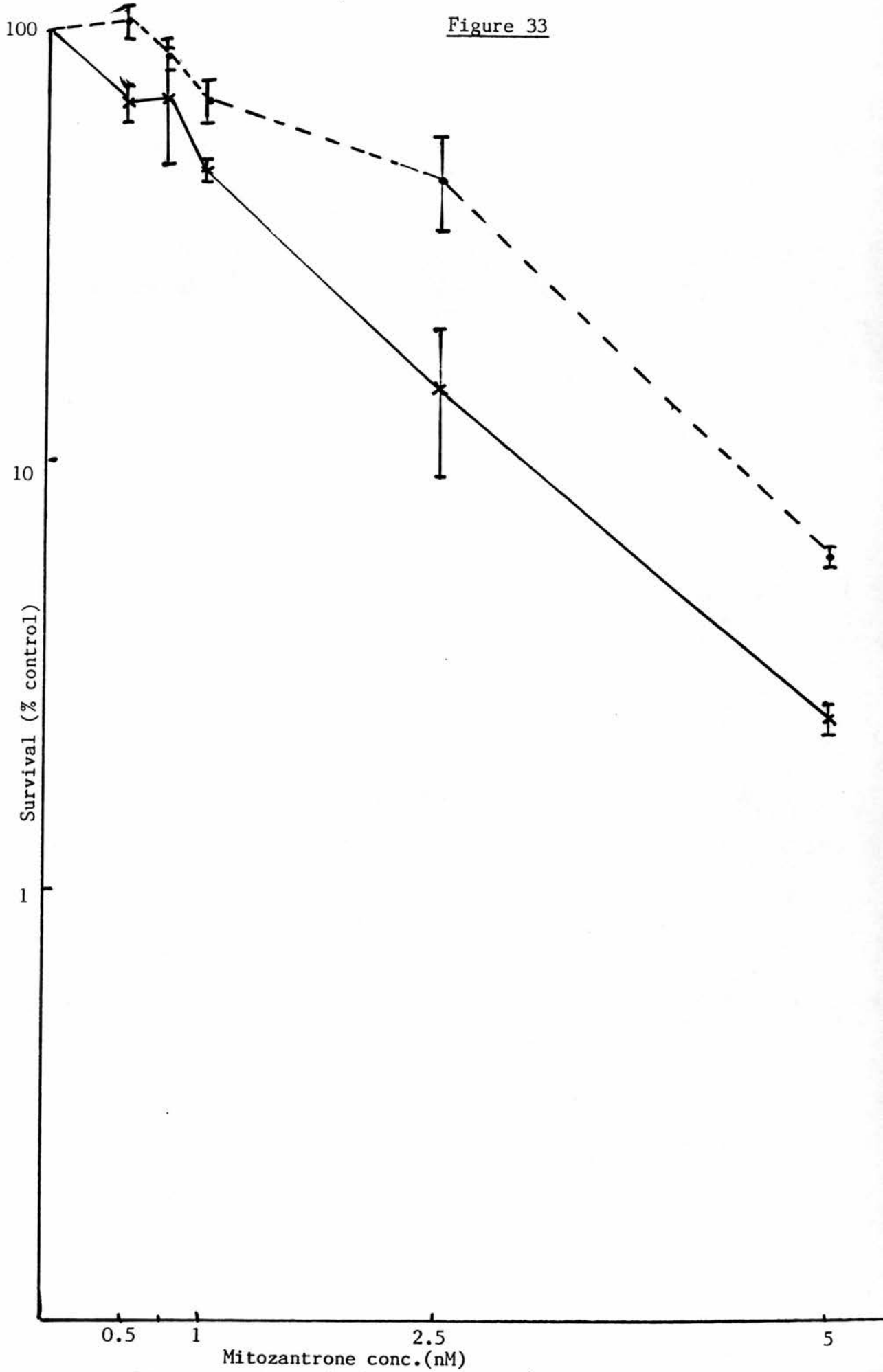


Figure 34 Prednimustine Sensitivity

PE/01 ×——×

PE/04 ●- - -●

PE/01 CisPt^R □—□

Dose (uM)	% Survival PE/01	PE/04	PE/01 CisPt^R
0.2	49.9 ± 17.2(3) (38.8)	-	-
0.5	37.8 ± 12.5(4) (39.8)	-	-
0.8	24.3 ± 8.8 (3) (27.5)	95.1 (1) (158.8)	78.1 (1)
1.0	13.4 ± 4.2 (4) (18.3)	77.9 ± 15.1(3) (110.4)	63.9 ± 7.7 (3) (56.4)
2.0	3.5 ± 2.5 (3) (2.5)	59.4 ± 11.2(3) (70.0)	56.8 ± 17.0(3) (55.3)
3.0	-	28.0 ± 3.6 (4) (33.4)	41.4 ± 9.1 (4) (43.1)
4.0	-	14.3 ± 2.4 (3) (11.6)	14.5 ± 3.3 (3) (14.3)
5.0	-	15.9 (1) (7.1)	16.4 (1) (14.8)
6.0	-	5.7 ± 3.4 (3) (4.3)	3.0 ± 1.4 (3) (2.5)

Notes as in figure 17.

Figure 34

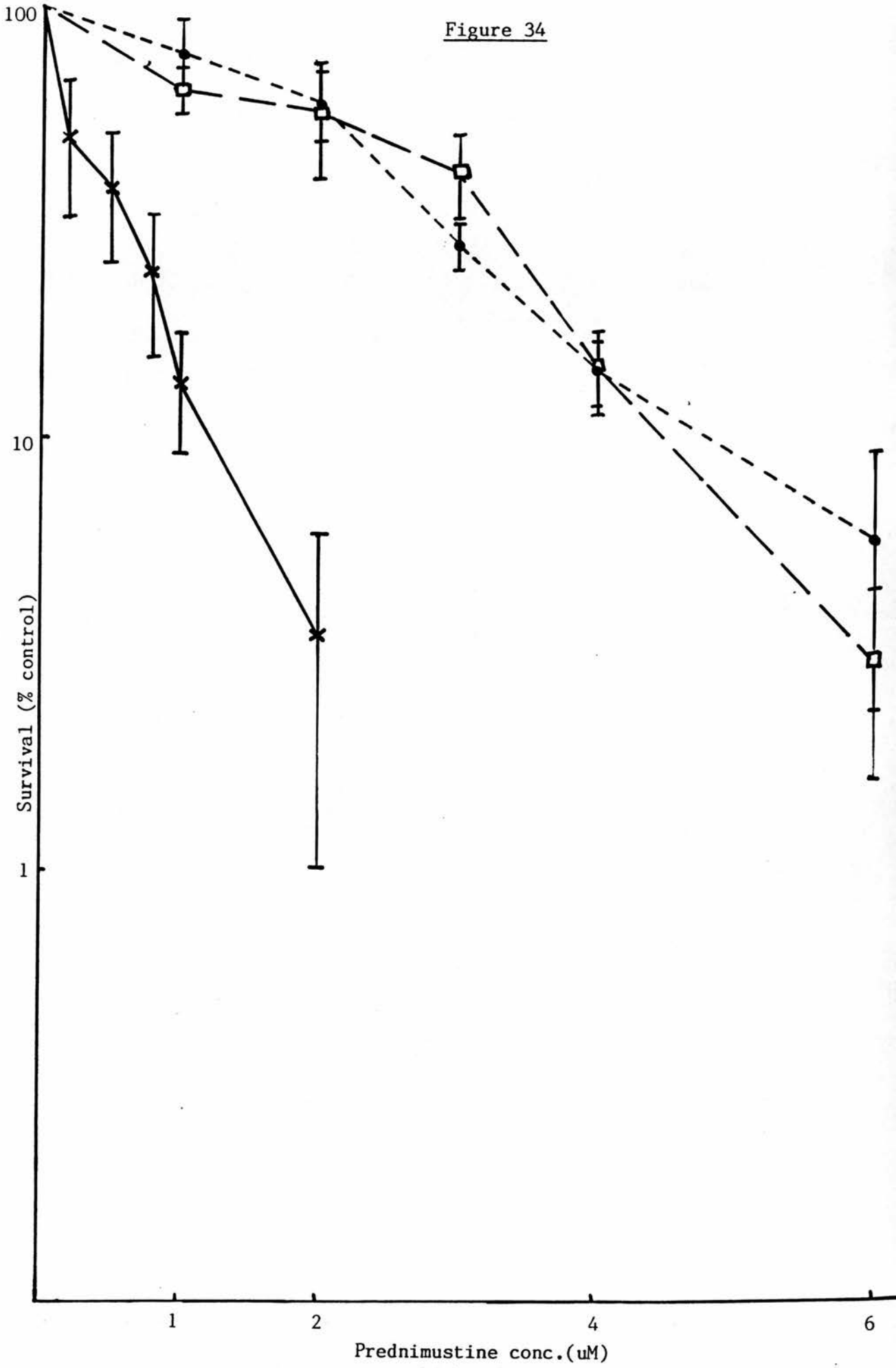


Figure 35 X-ray Sensitivity

PE/01 ×——×

PE/04 ●- - -●

PE/01 CisPt^R □—□

Dose (rads)	% Survival PE/01	PE/04	PE/01 CisPt^R
50	81.5 ± 5.8 (3) (86.3)	76.2 ± 2.7 (2) (84.9)	73.7 ± 8.5 (3) (84.7)
100	39.9 ± 5.1 (3) (46.9)	50.7 ± 0.6 (2) (73.6)	61.3 ± 5.7 (3) (81.0)
200	11.2 ± 3.2 (3) (16.4)	24.1 ± 6.0 (2) (14.4)	44.3 ± 8.7 (3) (51.9)
500	0.56± 0.15(2) (0.07)	0.67± 0.11(2) (0.3)	6.3 ± 1.1 (3) (10.2)
800	0.076±0.002(2) (0)	0.07± 0.07(2) (0)	0.74± 0.20(3) (1.5)

Notes as in figure 17.

Figure 35

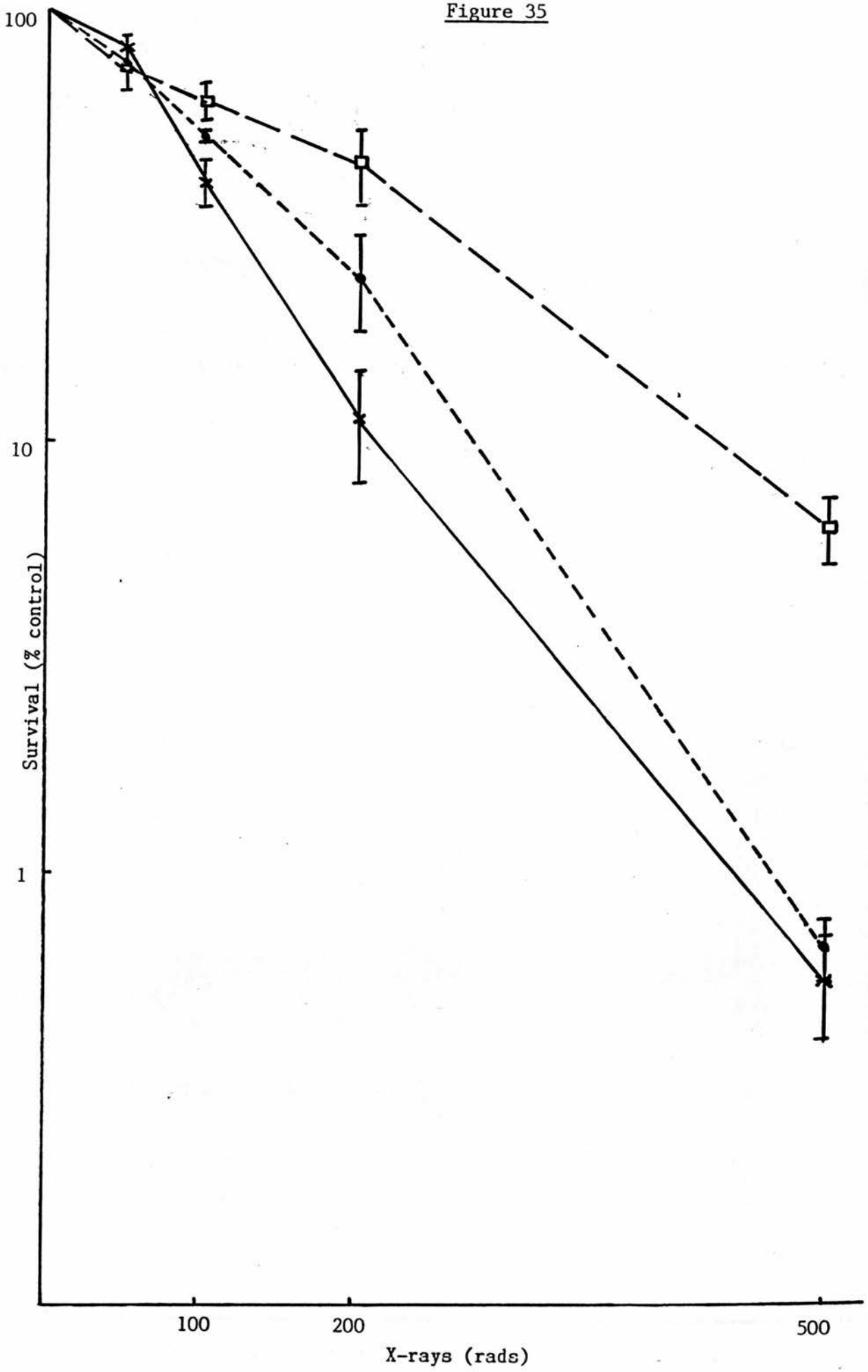


TABLE 10

LD_{50} VALUES FOR VARIOUS DRUGS[a]

	PE/01	PE/04		PE/01	$CisPt^R$
Cisplatinum	0.082uM	0.23uM	(3x)[b]	1.6uM	(25x)
CHIP	0.54uM	0.67uM		2.7uM	(5x)
CEDCA	1.0uM	2.1uM	(2.1x)	7.5uM	(7.5x)
JM40	0.22uM	2.0uM	(9x)	8.8uM	(40x)
Chlorambucil	0.83uM	2.8uM	(3.3x)	3.3uM	(4x)
Melphalan	0.31uM	1.3uM	(4.2x)	2.3uM	(7.4x)
5-fluorouracil	6uM	4uM		-	
Doxorubicin	7.0nM	18nM	(2.5x)	13nM	(2x)
Vincristine	3.5nM	5.6nM	(1.6x)		
Mitozantrone	1.0nM	2.0nM	(2x)	-	
Prednimustine	0.25uM	2.2uM	(9x)	2.4uM	(9x)
X-rays	84 rad	100 rad	(1.2x)	160 rad	(1.9x)

(a) 3 day exposure in clonogenic assay on plastic.

(b) approximate increase in resistance over PE/01 shown in brackets.

a combination of both. All the dose response curves were of the exponential or threshold-exponential type.

The PE/04 cell line was equally sensitive or more resistant than PE/01 to all the drugs tested. With the platinum analogues little difference between PE/01 and PE/04 in sensitivity to CHIP was observed, some small difference seen towards CBDCA (approximately 2-fold) but a larger difference shown towards JM40 (approximately 9-fold). This pattern of sensitivity was also reflected in the PE/01 CisPt^R cell line where resistance to cisplatinum was accompanied by high resistance to JM40 but considerably less resistance to CBDCA and even less to CHIP. All 3 analogues used here were toxic only at higher concentrations than cisplatinum itself. The sensitivity to melphalan showed a similar pattern to that for chlorambucil and PE/01 CisPt^R showed only some partial cross-resistance to melphalan. Experiments with melphalan have not been repeated at 5%O_2 in the gas phase as yet. Interestingly prednimustine showed a rather higher level of cross-resistance, showing a large difference between PE/01 and PE/04.

Very little cross-resistance was observed in PE/04 to the other unrelated drugs doxorubicin, vincristine and mitozantrone even though these drugs were toxic at nanomolar concentrations. PE/01 CisPt^R was also only

marginally cross-resistant to doxorubicin. PE/01 and PE/04 had a similar sensitivity to x-rays with PE/04 CisPt^R showing some small increase in resistance relative to its parent cell line.

3.5 PE/04 CisPt^R and PE/06 cell lines

These 2 cell lines which have been derived more recently (see chapter 2) have both been tested against cisplatinum in the clonogenic assay on plastic.

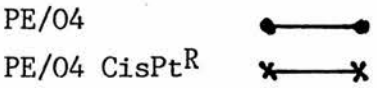
PE/04 CisPt^R

The results from 1 experiment testing the sensitivity of the cell line to cisplatinum are shown in Figure 36 together with the sensitivity of PE/04 tested at the same time. In this assay PE/04 CisPt^R (passage 73) was tested after passaging until then in 1uM cisplatinum. Subsequent passaging in the absence of the drug (from passage 75) is continuing to see if the resistance will be maintained. At passage 73 PE/04 CisPt^R was some 4.4 fold resistant to cisplatinum compared with PE/04 and its resistance was at a very similar level to that of PE/01 CisPt^R, perhaps not surprisingly since both were selected in 1uM cisplatinum.

PE/06

PE/06 (line derived in 20% O_2) at passages 7 and 9 was tested against cisplatinum and showed a dose response as shown in Figure 37 with an LD_{50} of approximately 0.3uM, similar to PE/04. Although further experiments are needed, this would confirm the difference in sensitivity

Figure 36 PE/04 CisPt^{R} sub-line - Cisplatinum Sensitivity



Both cell lines tested at the same time in the clonogenic assay on plastic. Bars represent standard error of the mean of triplicates in the one experiment. Dose left on for 3 days.

Figure 36

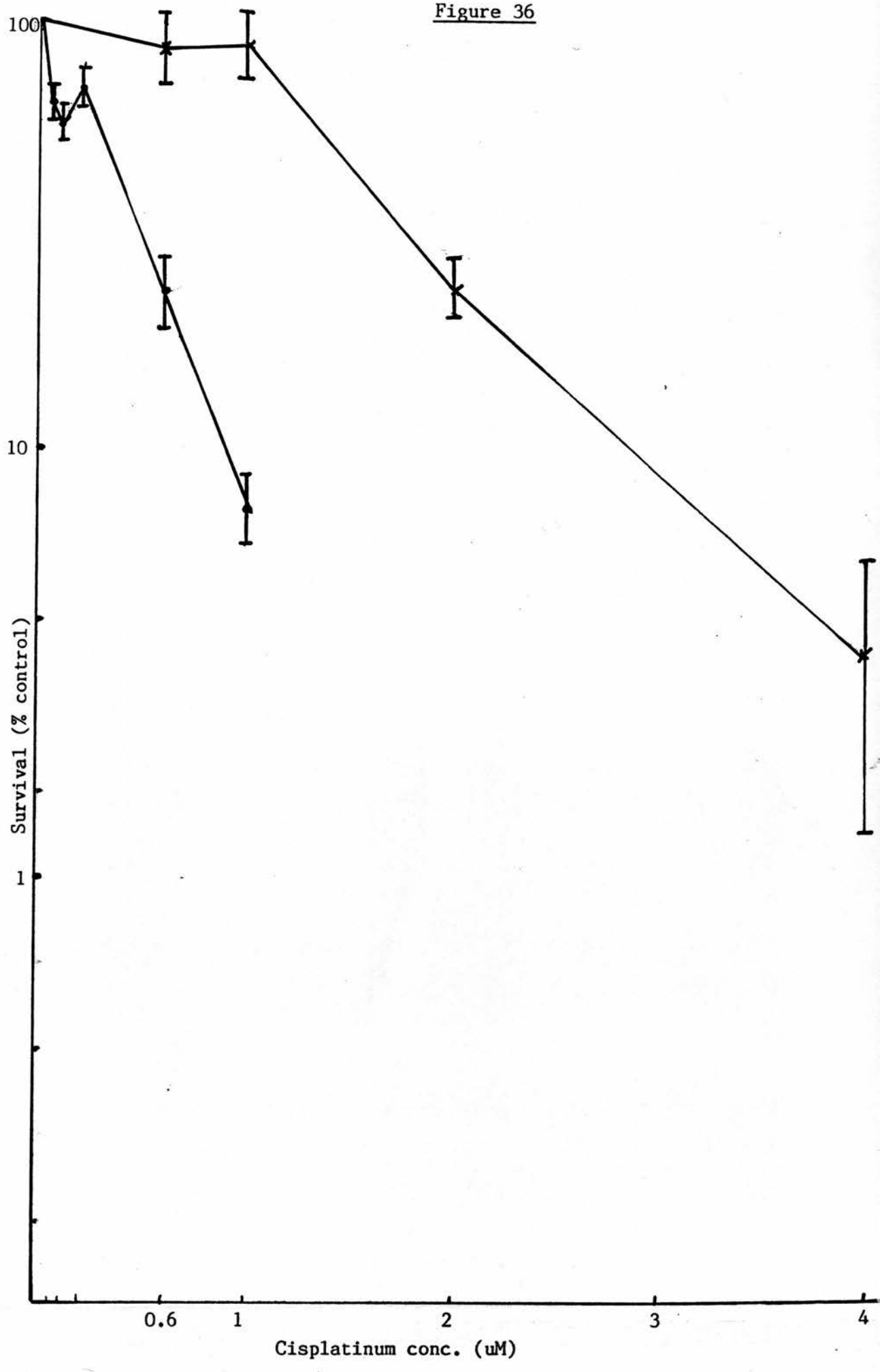
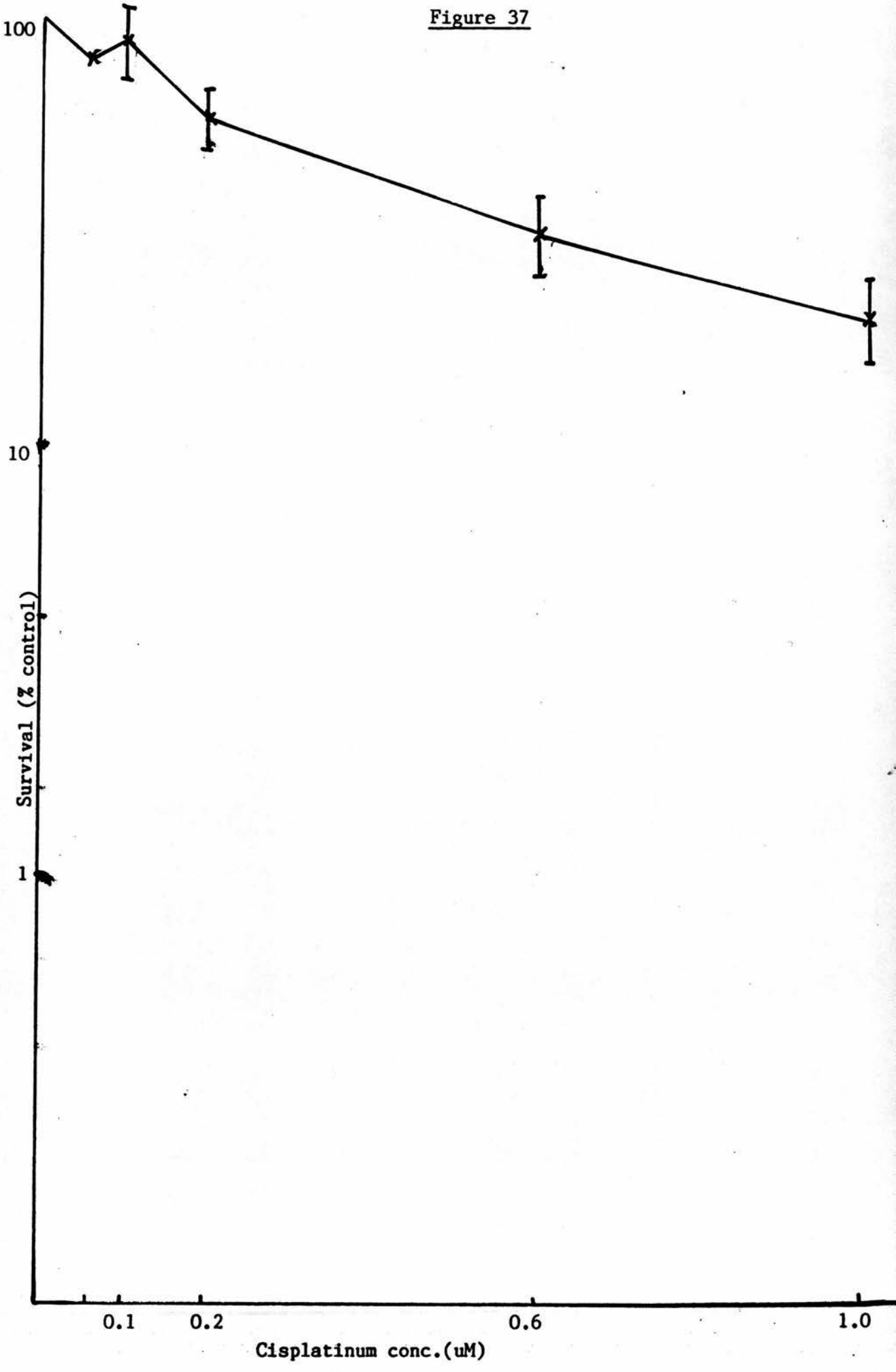


Figure 37 PE/06 - Cisplatinum Sensitivity

Sensitivity in clonogenic assay on plastic. Dose left on for 3 days.
Mean and standard error from 2 experiments.

Figure 37



between the cell lines from ascites taken after the patient's treatment (PE/04 and PE/06) and the cell line derived from ascites taken upon relapse just before the courses of chemotherapy (PE/01).

3.6 Discussion

PE/04 shows a marked resistance to cisplatinum compared with PE/01 in both the assays used. Resistance to chlorambucil occurred in only one assay and could be altered by changing the oxygen tension. Its significance is thus much more open to question and its relevance to the phenotypic properties of PE/04 is unclear since undoubtedly cisplatinum resistance with cross-resistance to classical bifunctional alkylating agents must be considered separately to cisplatinum resistance without such cross resistance (A. East man et al, 1981). The PE/01 $CisPt^R$ line shows cisplatinum resistance without much cross resistance to the alkylating agents chlorambucil and melphalan so it will be interesting to see what specific biochemical changes from the PE/01 phenotype, compared with the changes in PE/04, have occurred in it. PE/04 showed high resistance to prednimustine although PE/01 $CisPt^R$ showed no greater resistance. The toxicity of prednimustine is apparently due to its slow hydrolysis to chlorambucil thus prolonging the availability of the toxic chlorambucil species (B. Hartley-Asp et al, 1986). From the LD_{50} values it may be

that PE/01 is more sensitive to prednimustine than chlorambucil rather than PE/04 more resistant, perhaps due to the prolonged availability of chlorambucil derived from the prednimustine. Cross resistance to other drugs like doxorubicin and vincristine involved in the pleiotropic resistance phenotype (J.H. Gerlach et al, 1986) appears to be minimal.

Various assay conditions have been shown to affect drug sensitivity measurements although the evidence from different groups can be contradictory (B.T. Hill, 1983). Provided the conditions of drug exposure are the same, other aspects of the assay method may not change the apparent drug sensitivity very much (B.T. Hill et al, 1983). The metabolic state of the cells when treated - either in suspension after trypsinisation or after attachment to a growth surface - has been shown to be important (P.R. Twentyman, 1979) and other factors such as anchorage dependence (T.C. Stephens et al, 1980), oxygen tension (V. Gupta et al, 1982) and the addition of rat red blood cells (L. Endresen et al, 1985) have all affected drug sensitivity determinations in some systems. The effects of cell density or cell transfer after drug exposure can be significant and affect the sensitivity to some drugs more than others (P.J. Hepburn et al, 1986). In the experiments shown here the drug sensitivity to chlorambucil has not altered very much in terms of the

concentration of drug required but the relative sensitivity of 2 closely related cell lines has changed from being obviously different to minimally if at all different. More experiments with low oxygen tension will clearly be interesting especially with melphalan to compare with chlorambucil and PE/01 CisPt^R to compare with PE/01 and PE/04. Whether the amount of cross-resistance observed with melphalan or prednimustine would be reduced at 5%O_2 in a similar manner to chlorambucil needs to be determined.

It would appear that 5-fluorouracil is less toxic in the clonogenic assay on plastic than in the Courtenay assay. Possibly the increased time of drug exposure in the Courtenay assay (21 days versus 3 days) could result in this increased toxicity since the length of exposure time is known to be important (A.F. Sobrero et al, 1983), or it may simply be a result of the different conditions of the two assays. The variation is presumably caused by variation in nucleoside concentrations in different batches of serum (or red blood cell preparations). Hams F-12 medium used in the Courtenay assay already contains 3uM thymidine, which is thought to be a principal modulator of 5-fluorouracil metabolism (B. Ardalan et al, 1980) but RPMI 1640 medium used in the clonogenic assay on plastic contains no nucleosides.

No changes in drug sensitivity with passaging the cell lines in vitro have been observed. In recent work with the Courtenay assay we specifically returned to as early passages as possible to check this aspect. While some authors have noted changes in drug sensitivity with passaging (A.P. Wilson, 1984; F. Holzel et al, 1985) others have shown remarkably little change in drug sensitivity with passaging (K.M. Tveit et al, 1981b) even when other changes in the cell line have occurred (P.G. Parsons et al, 1982) or over very long periods in culture (J.R.W. Masters et al, 1986). An additional reason for returning to early passage cells was as a link in checking any changes back to the ascites itself. Problems with cell clumping have been reported by many authors with agar clonogenic assays (S. Rockwell, 1985) and this has been a problem with our ascites preparations. Various methods of disaggregation especially using different enzymes such as collagenase, dispase and DNAase are presently under investigation. A collagenase/DNAase mixture appears to be effective with PE/04 ascites.

A number of authors have attempted assays with radiolabelled compounds particularly [^{3}H]-thymidine (Group for Sensitivity testing of tumors, 1981) and [^{3}H]-leucine (S. Merry et al, 1984). The validity of these various assays has been recently reviewed (R.I. Freshney et al, 1983; L.M. Weisenthal, 1981). Correlations with

clonogenic assays have been observed (G.E. Johnson et al, 1983) although sometimes with considerable scatter (N. Tanigawa et al, 1982) and appear better in assays over several days allowing recovery after drug treatment than in short term assays conducted over a few hours (A.P. Wilson et al, 1984; K. Zirvi et al, 1986). Short term assays may only show a qualitative comparison (H.T. Rupniak et al, 1983) although clinical correlations have been observed (B.T. Hill, 1983a). Others have shown spurious resistance with radiolabel incorporation assays at shorter times with sensitivity increased once a recovery period was allowed (D. Morgan et al, 1983; C. Roobol et al, 1984). Radiolabelling at the time of colony counting in a clonogenic assay seemed to give good correlations (P.G. Parsons et al, 1979; C. Roobol et al, 1984) as observed in this project.

The amount of variation in drug sensitivity which is regarded as biologically significant is also an important question. Many studies have used cell lines with resistance acquired in vitro to very high levels over 100-fold (V. Ling, 1982). However in the clinical setting it is clear that resistance of 3-4 fold would often be enough for a tumour to survive therapy (E. Frei et al, 1980) and high dose cisplatinum therapy has shown responses in patients with ovarian cancer who failed standard dose therapy (R.F. Ozols et al, 1985). Thus changes in drug

sensitivity of the order of magnitude shown with the cell lines here would be important clinically. Similar levels of in vitro acquired resistance are thought to be significant (T.C. Hamilton et al, 1984a). High stable levels of resistance (above about 10-fold resistant) have been difficult to produce in vitro to agents such as BCNU, nitrogen mustard (HN2) and cisplatinum (E. Frei et al, 1985), and melphalan (P.G. Parsons et al, 1978). A similar degree of variation (5-fold) in sensitivity to cisplatinum has been observed in clonal human glicma cell lines from a single untreated tumour (W-K.A. Yung et al, 1982) which serves to emphasise the importance of tumour heterogeneity as far as drug sensitivity is concerned (T. Tsuruo et al, 1981) particularly in vivo and this phenomenon has been observed in primary ovarian cancer samples (J. Siracky, 1979). Although heterogeneity makes predictions on the response of a primary tumour in vivo from results in vitro on a cell line derived from it difficult, such correlations have been observed with ovarian tumours and cell lines (22 cases, M. Albrecht et al, 1985). The preliminary data with PE/06 giving a similar response as PE/04 to cisplatinum (and resistant compared with PE/01) would support the idea of a change induced by the chemotherapy in the patient.

The LD_{50} values reported here (Table 10) for PE/01 and PE/04 are at the lower end of the range for cisplatinum

toxicity values (approximately 0.03-3uM for a 24 hour to continuous dose) reported in the literature for ovarian (F. Holzel et al, 1985; R.N. Buick et al, 1985; L.M. Van Putten et al, 1986) and other human cell lines (B.T. Hill et al, 1984) and below that reported for normal human bone marrow cells (0.9uM, G.E. Umbach et al, 1985) or the concentration commonly used in the Hamburger/Salmon clonogenic assay for ovarian cells (0.7uM) at 1/10th the achievable peak plasman level (D.D. Von Hoff et al, 1983; D.S. Alberts et al, 1980a). However a 1 hour treatment is often used in the Hamburger/Salmon assay and the LD_{50} values for a 1 hour cisplatinum treatment on PE/01 and PE/04 (approximately 1.1uM and 3.3uM) are a little above this value of 0.7uM although still well in the range (0.7uM-10uM) reported in the literature for ovarian cell lines (G. Clamon et al, 1985; J. Benard et al, 1985; A.C. Jones et al, 1984; L.M. Van Putten et al, 1986) or other human cell lines (L. Endresen et al, 1985).

Various authors have shown increased cytotoxicity of cisplatinum against human cells in tissue culture with the time of incubation (J.P. Bergerat et al, 1979; H.T. Rupniak et al, 1983a; H. Hisazumi et al, 1983; E. Cadman et al, 1984; A.F. Sobrero et al, 1985) suggesting that toxic species are present at least for several hours. Bergerat suggested a simple concentration x time computation would indicate the effective dose and that

little cytotoxic activity is lost in tissue culture medium containing 10% foetal calf serum over 24 hours. However when LD_{50} doses at various incubation times from the other references are computed as a concentration x time value, the 1-4 hour values are generally less than the 24 hour values suggesting decomposition to non-cytotoxic species in this time. Recent data by Hill (B.T. Hill et al, 1985) has reported that the difference between a 1 hour and 24 hour equitoxic dose depends on the cells being tested and can vary from 6 to 50 fold.

Cisplatinum reacts with serum proteins to produce non-cytotoxic species (K. Takahashi et al, 1985) and so will decompose in the culture medium used here (see also the discussion on cisplatinum in chapter 1). Although cisplatinum has a half-life of some one-and-a-half hours in human plasma in vitro (A.J. Repta et al, 1980) it is unclear how much of this decomposition is to non-cytctoxic species. Holdener reported substantially reduced cytotoxicity against bone marrow cells in vitro after pre-incubation of cisplatinum in human plasma for 4 hours and even less cytotoxicity after 24 hours pre-incubation (E.E. Holdener et al, 1983). Decomposition also occurs in plasma ultrafiltrates in vitro at similar rates despite the presence of high chloride concentrations (100mM) which inhibit decomposition in simple buffers (A.J. Repta et al, 1980) and at least 7 low molecular weight platinum

containing species have been observed (P.T. Daley-Yates et al, 1984). These species are formed in different proportions in vitro from in vivo experiments with plasma. In rat plasma in vitro 85% of the platinum is protein bound after 24 hours ($t^1/2$ for protein binding 57 minutes), parent cisplatinum is present in only trace amounts and the major ultrafilterable species (44%) was tentatively identified as the cisplatinum hydrolysis product (P.T. Daley-Yates et al, 1984). In tissue culture medium containing 10% foetal calf serum similar decomposition and reaction with proteins would be expected although perhaps at a reduced rate to that in 100% serum or plasma. RPMI-1640 medium contains 100mM chloride ion concentration similar to that in plasma but concentrations of other low molecular weight species will be different. Platinum binding to bovine serum albumin (0.69gm/100mls) in saline occurs at similar rates to protein binding in plasma (A.F. Leroy et al, 1979) and albumin is the major protein in foetal calf serum (2.6gm/100mls of the total 4.5gm/100mls protein present; Gibco Quality Control Information).

The toxicity of chlorambucil reported here is at the low end of the range (2.4-13.5uM) reported for melanoma cell lines (P.G. Parsons et al, 1979) but similar to that for normal human fibroblasts (P.G. Parsons et al, 1979) and greater than that commonly used in the Hamburger/Salmon

assay (0.3uM). For 5-fluorouracil the values are similar to other human cell lines (range 1-23uM B.T. Hill et al, 1984; F. Holzel et al, 1985) and normal bone marrow cells (2.8uM, G.E. Umbach et al, 1985) and concentrations used in the Hamburger/Salmon assay (6uM). The exposure time has been shown to be important for the activity of many antineoplastic drugs in vitro (Y. Matsushima et al, 1985) particularly for antimetabolites but apart from cisplatinum was not further pursued in this project.

All three of the platinum analogues were less toxic than cisplatinum. This has been the common experience of other authors both in vitro (A.C. Jones et al, 1984; B. Drewinko et al, 1985) and in vivo (E. Boven et al, 1985). Jones observed approximately 10-fold less toxicity with CBDCA than cisplatinum and Boven noted maximally toxic doses to xenograft bearing nude mice at 5mg/kg for cisplatinum, 60mg/kg for CBDCA and 40mg/kg for CHIP and JM40. In the clinical setting this may not be critical since higher doses can be given. In phase I-II studies doses have been up to 350mg/m^2 for CHIP (V.H.C. Bramwell et al, 1985), 550mg/m^2 for CBDCA (R.A. Joss et al, 1984) and 1200mg/m^2 for JM40 (B. Winograd et al, 1986). In refractory ovarian cancer Ozols and his colleagues have used 40mg/m^2 qd x 5 cisplatinum versus 400mg/m^2 qd x 2 CBDCA (R.F. Ozols et al, 1985a). The lack of cross resistance of CHIP and to a lesser extent CBDCA is the most interesting fact in the

present study and needs to be pursued since both these drugs are of clinical interest in ovarian cancer. To date CBDCA has received most clinical interest and its current status and prospects have been extensively reviewed recently (S.K. Carter et al, 1985). It has shown similar activity to cisplatinum in ovarian cancer with a response rate of approximately 20-25% in patients pretreated with cisplatinum (E. Wiltshaw, 1985; R. Canetta et al, 1985). CHIP has shown some responses in ovarian cancer patients pretreated with alkylating agents (V.H.C. Bramwell et al, 1985). The potential for differential activity of the analogues, quite apart from their differential toxicity to normal tissue (P. Lelieveld et al, 1984), needs to be further explored. Earlier studies have explored the structure-activity relationships of many analogues in rodent tumours (K.R. Harrap, 1983). Different analogues have shown varying degrees of cross-resistance to L1210 cells resistant to the parent drug cisplatinum (no cross-resistance up to 83-fold cross-resistance in a 50-fold cisplatinum resistant sub-line, J.H. Burchenal et al, 1979). Bis-(isopropylamine)dichloroplatinum a Pt(II) analogue similar in structure to the Pt(IV) analogue CHIP showed only a limited 7-fold resistance. In vivo studies with cisplatinum resistant L1210 cells have suggested cross-resistance between all three of the analogues used here and cisplatinum (W.C. Rose et al, 1984). However the clinical data (R. Canetta et al, 1985) and the data

presented here do not support the conclusion cf complete cross-resistance and suggest further investigatiens with human tumours are warranted.

4. Mechanisms of resistance to Cisplatinum - Cellular Uptake studies

The transport of cisplatinum into cells has generally been thought to be by passive diffusion of the unreacted neutral complex (B. Rosenberg, 1985), based on the work of Gale with tritiated cis-dichloro(dipyridine)platinum II (G.R. Gale et al, 1973) where amongst, other characteristics consistent with passive diffusion, binding to Ehrlich tumour cells was virtually unimpeded at 0°C. Thus most work concerned with mechanisms of resistance has concentrated on intracellular events (see chapters 5 and 6). However, resistance at the membrane level has been shown to be an important mechanism with other drugs (G.A. Curt et al, 1984; G.J. Goldenberg et al, 1984) and I thought it worthwhile to check this aspect with cisplatinum particularly if cross-resistance to other alkylating agents like melphalan was important and as a matter of completeness in looking at the possible mechanisms of resistance a cell could display. Resistance to melphalan in a L1210 cell line, which was also cross-resistant to cisplatinum (F.M. Schabel et al, 1978) has been associated with changes in a transport mechanism for melphalan (W.R. Redwood et al, 1980). However, Eastman has also reported a L1210 sub-line specifically resistant to cisplatinum without such cross-resistance (A. Eastman et al, 1981). Melphalan is known to enter cells by

carrier mediated transport particularly via the carrier system for the amino acid leucine (A. Begleiter et al, 1979) and resistance with a defect in this mechanism has been shown in chinese hamster cells (A.H. Dantzig et al, 1984). There is an extensive literature regarding the transport of alkylating agents (G.J. Goldenberg et al, 1984). In addition Byfield has classified alkylating agents (using the term in its broadest sense) into those with carrier dependent or carrier independent transport (J.E. Byfield et al, 1981) on the basis of water soluble alkylating agents being more toxic to phytohaemagglutinin stimulated human lymphocytes than resting lymphocytes while lipid soluble agents were not. This division held for a wide variety of agents including melphalan, cisplatinum, nitrosoureas and mitomycin C and also extended to x-rays. He suggested cisplatinum was carrier-dependent and probably transported into cells by an amino acid transport mechanism as amino acids protected the lymphocytes. Some support for this claim has come from the report of Scanlon who showed inhibition of sodium-dependent amino acid transport in L1210 cells by cisplatinum (K.J. Scanlon et al, 1983). However when this project began there was no direct evidence for any association of transport effects with sensitivity to cisplatinum or in acquisition of resistance to it. Some evidence has been put forward since then as discussed at the end of the chapter.

The quantitation of the amount of cisplatinum in a particular system has generally relied on atomic absorption spectroscopy to measure the platinum after separation of different platinum containing species where appropriate and these procedures have been used in most pharmacokinetic studies (C.L. Litterst et al, 1976; J.B. Vermorken et al, 1984). Some studies have used radioactive isotopes of platinum either ^{193m}Pt or ^{195m}Pt incorporated in cisplatinum (R.C. Manaka et al, 1980). These studies have been limited by the short half-lives of these isotopes (4.3 and 4.1 days respectively). Only relatively few studies have looked at uptake into cells in vitro (J. Uozumi et al, 1984) and I chose to try and measure the amount of platinum taken up into cells by atomic absorption spectroscopy (AAS). Later in this project I was able to use some ^{195m}Pt cisplatinum available through Dr. Philip Bedford at I.C.R.F. in London to confirm the AAS work. No attempt was made to measure actual transport processes but rather I measured total cellular platinum at various time points starting at 1 hour after cisplatinum was added to the incubation.

Some preliminary experiments using scanning electron microscopy with x-ray microanalysis to detect electron-dense platinum taken up by cells were attempted. Two papers have reported nucleolar localization of platinum by this method but at high cisplatinum concentrations (200uM

cisplatinum on HeLa cells, M.U.A. Khan et al, 1978; 1.6mM cisplatinum on human fibroblasts, J.P. Berry et al, 1983). Others have used this technique to localize platinum in liver and kidney after cisplatinum administration to rats (T. Makita et al, 1985). However in another report platinum could not be detected in HeLa cells after exposure to 3-30uM cisplatinum in vitro (F.N. Ghadially et al, 1981). I could detect 0.4nmoles cisplatinum dried down on to a carbon stub but platinum could not be detected in rat gut samples after in vivo cisplatinum treatment (S.G. Allan, unpublished results) and the technique was not pursued further. The results at very high cisplatinum concentrations are of doubtful biological significance and others have suggested that atomic absorption spectroscopy is much more sensitive when the element being analysed is highly dispersed in the nucleus or cell (C.F. Shaw et al, 1981) as is most likely the case after cisplatinum treatment (R.P. Sharma et al, 1983).

4.1 Methods

4.1.1 Atomic Absorption Spectroscopy

Initial contact with Dr. A. Rowley in the Department of Chemistry, University of Edinburgh quickly showed that flame atomic absorption spectroscopy was unsuitable due to interference by sodium chloride present in biological samples. Non-flame atomic absorption spectroscopy has

been successful (R.P. Sharma et al, 1983a) and preliminary work was done using a graphite furnace spectrometer in the Department of Applied Chemical Sciences at Napier College, Edinburgh (with the help of Ms. D. Jeffrey Smith). Since obtaining time on this machine was difficult further work was done at the Department of Clinical Biochemistry, Royal Infirmary, Glasgow (with technical advice there from Dr. D. Halls). Advice on the technique and sample preparation was also obtained from the Department of Applied Chemistry, Strathclyde University, Glasgow (Dr. D. Littlejohn and Professor J. Ottaway).

Approximately 10^7 PE/01 and PE/04 cells grown in $75cm^2$ flasks (Nunc, Gibco Ltd) were exposed to various concentrations of cisplatinum and incubated in a high humidity 5% CO_2 incubator at 37^oC for times ranging up to 24 hours. The cells were then harvested by trypsinisation. Flasks were taken from the incubator, cooled quickly (3 minutes in a -20^oC refrigerator), and rapidly washed (less than 10 seconds) twice with ice-cold PBS before trypsin (2.5mls) was added. When cells had detached, 5mls of RPMI-1640 (without serum) was added and the cells in this medium were counted and transferred to soda glass tubes (Samco) and dried down at 80^oC. The dried down residue was digested overnight in 2mls concentrated nitric acid heated to 80^oC and after cooling 50uls of 30% hydrogen peroxide solution added to aid

dissolution of any remaining residue (similar procedure to R.P. Sharma et al, 1983a). This solution was then analysed for platinum by atomic absorption spectroscopy. This harvesting and digestion procedure was designed to minimise any loss of unbound platinum from the cells by washing the medium at low temperatures and collecting all the trypsin solution not just a cell pellet after centrifugation. This washing procedure should leave 2.5ng platinum equivalent or less due to cisplatinum left from the medium from a 10uM cisplatinum incubation.

Platinum was analysed on a Perkin Elmer 2280 atomic absorption spectrometer with pyrolytic coated graphite tube furnaces and a HGA 500 programmer. The instrument parameters used are shown in Table 11 and were modified from those of J. Haron (PhD thesis, University of Strathclyde, 1982). An autosampler was used to inject 20ul aliquots into the graphite furnace. Digested samples in nitric acid were diluted 1:1 with water, which contained a standard addition of cisplatinum (usually 0,40,80 and 120ng/ml cisplatinum), in the autosampler cup before the analysis and 2 to 3 aliquots were injected and analysed to confirm the reading obtained. Values in fgm platinum equivalent/cell were calculated from the sample reading and 3 different standard additions of cisplatinum.

Preliminary experiments showed decreases in the atomic

TABLE 11

INSTRUMENT PARAMETERS FOR PLATINUM

ATOMIC ABSORPTION SPECTROSCOPY

Furnace Programme	Ramp	Hold	Temperature
Drying stage	1 sec	10 sec	100°C
	5 sec	20 sec	180°C
Ashing stage	5 sec	40 sec	1500°C
Atomisation stage	0 sec	10 sec	2700°C
Cleaning stage	0 sec	3 sec	2700°C

Internal gas flow during atomisation 10ml/min

Wavelength 265.9nm

Lamp current 30.0

Background corrector ON, Autozero 2 seconds before atomisation stage.

absorption signal with increased nitric acid concentration and increased cell number in the sample. For these reasons measuring the signal with a standard additions procedure was preferred to simply using a standard reference curve (R.P. Sharma et al, 1983a) especially since sample matrix effects are known to be important parameters in interference with the atomic absorption peak and aging of the carbon tube furnace was also experienced (A.J. Repta et al, 1980). The signal was more reliably measured by peak height than by the integrated peak area due to negative tails to the peak, probably caused by the deuterium arc background correction. Pyrolytic coated graphite tubes gave improved sensitivity. Some precipitation was observed in the digested sample solutions which could be dissolved by addition of hydrogen peroxide but later reappeared. It could be redissolved on simply heating the solutions to 50°C. Attempts to evaporate off the nitric acid (at 125°C) and redissolve the residue in 0.1M HCl still left some precipitate and this procedure was not subsequently used. More elaborate digestions, for example, by heating with perchloric acid were beyond the facilities of this laboratory and the digestion with concentrated nitric acid was thought to be sufficient for the purpose.

The methodology of atomic absorption spectroscopy for platinum has been the subject of considerable debate in

the literature in regard to sample preparation including digestion and possible derivatization of products, the presence of sample matrix effects, interference by various ions and acids like nitric acid, and carbon furnace conditions and instrument parameters (A.F. LeRoy et al, 1977; M.F. Pera et al, 1977; S.J. Bannister et al, 1979; J. Smeyers-Verbeke et al, 1981; D. Priesner et al, 1981; R.P. Sharma et al, 1983a). Various authors have reported different sensitivities from 0.055ng to 3.8ng platinum without it being clear why there should be so much variation (J. Haron, 1982; and references above).

4.1.2. Methods - [^{195m}Pt] cisplatinum uptake

[^{195m}Pt] cisplatinum in 0.9% saline (1.152mg/ml cisplatinum, 0.58mCi/ml [^{195m}Pt] platinum) was obtained from Dr. P. Bedford and Dr. B.T. Hill at the I.C.R.F. laboratories in London. This was part of a batch prepared by Dr. H. Sharma, Department of Medical Biophysics, University of Manchester Medical School and I am grateful for the gift of this compound. [^{195m}Pt] platinum is a γ-ray emitting radionuclide with half life of 4.1 days which limits its use.

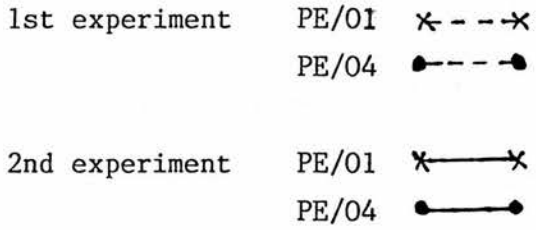
Cells from the PE/01 family of cell lines were harvested from log phase cultures and 5 x 10^6 cells taken up in 5mls RPMI-1640 without serum and incubated in suspension with

[^{195m}Pt] cisplatinum for 1 to 4 hours at room temperature. The cells were then spun down, washed twice with PBS and resuspended in 2mls PBS and counted for 1 minute on a γ-counter with windows set at 15-150 KeV. This washing procedure should leave at most 1ng/ml cisplatinum from the medium. All procedures were done with appropriate lead-brick shielding and monitoring in the Radioisotope Section of the Department of Medical Physics, Western General Hospital and I acknowledge the advice of Dr. J. Hannan of that Department. The cellular platinum concentration achieved was calculated from the counts obtained by reference to a standard curve of [^{195m}Pt] cisplatinum from the same stock and counted at the same time.

4.2.1 Results - Atomic Absorption Spectroscopy

Results from 3 experiments are reported here. Two experiments show the increase in the amount of cellular platinum in PE/01 and PE/04 cells over a 24 hour time course with 5uM cisplatinum in the medium (Figure 38). One flask of cells from each cell line was harvested for each time point. In the first experiment the platinum analysis included only one standard addition at 100ng/ml cisplatinum and the second drying stage in the instrument procedure was not used. Hence the second experiment is more reliable but wide variation between the two experiments can be seen. This is most probably due to

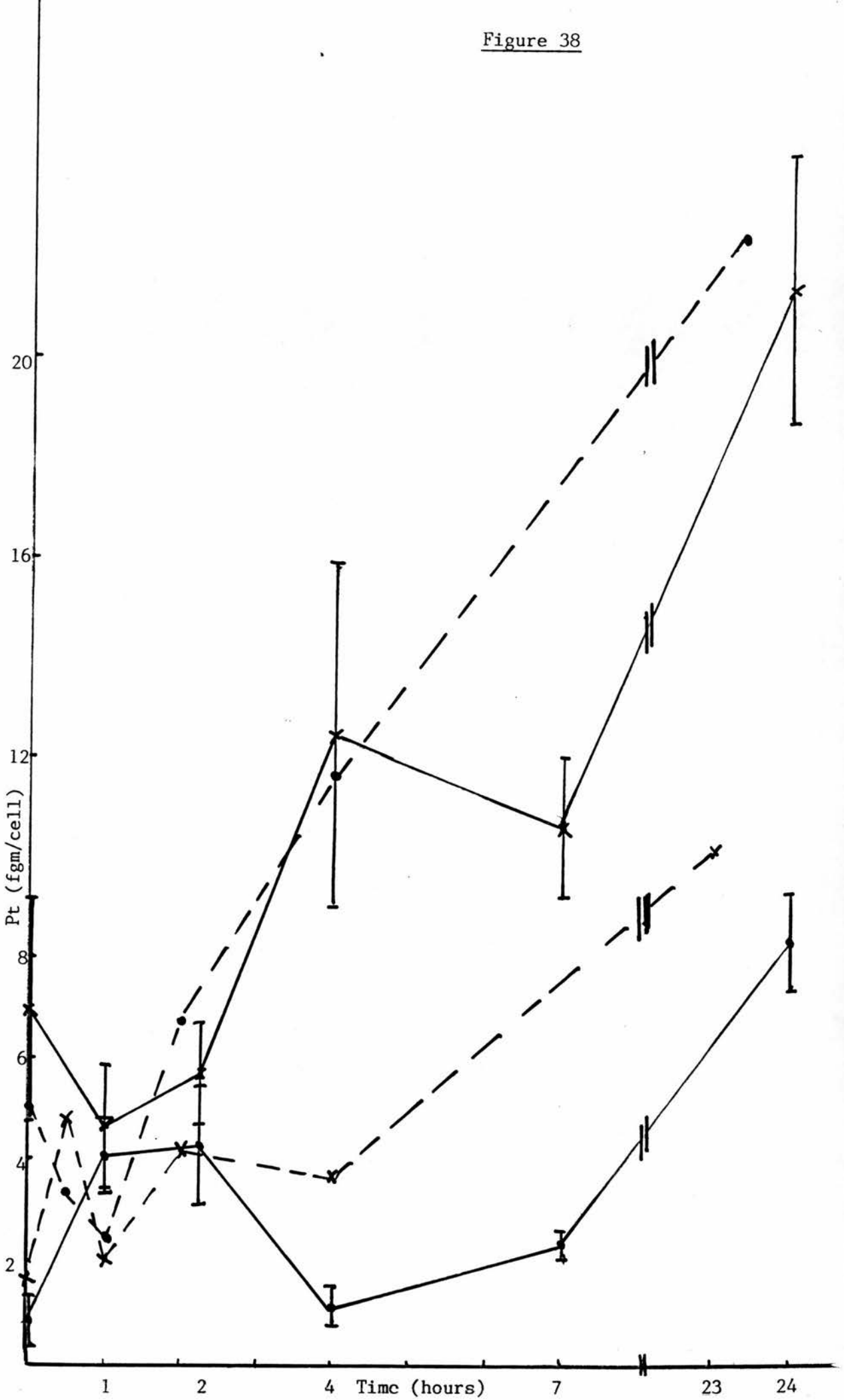
Figure 38 Time course of Cisplatinum uptake into PE/01 and PE/04



Cells in monolayer culture were exposed to 5uM cisplatinum in normal medium and harvested at various times up to 24 hours later and processed for measurement of total cellular platinum by atomic absorption spectroscopy.

Each point represents treatment on one flask of cells. The standard error bars for points in the 2nd experiment are calculated from linear regression analysis on the sample and three standard additions of cisplatinum.

Figure 38



working near the limits of sensitivity of the technique and particularly in the early time points absorbance readings were low, equivalent to approximately 20ng platinum/ml, and the peaks sometimes complex rather than singlets. However this concentration of platinum could be detected and quantitated in the use of the lowest standard addition of 40ng/ml (diluted 1 in 2 in the autosampler cup).

To assess whether there was a real difference in the platinum taken up into PE/01 and PE/04 cells a third experiment was done at the 24 hour time point where the platinum concentration was highest and possible background effects least. Duplicate flasks from both cell lines were incubated with 1, 5 and 10uM cisplatinum and the cells harvested and analysed as above. The flasks incubated with 1uM cisplatinum showed barely detectable platinum levels, the signal being within the background noise for 1 flask of PE/01. At 5uM and 10uM cisplatinum a platinum signal was readily detected. The results are shown in Table 12. Two flasks of cells not incubated with cisplatinum were harvested and the cell sample in RPMI-1640 spiked with cisplatinum (at 60 or 100ng platinum equivalent) before being dried down and processed with the other samples. The level of platinum detected was 94.7 $\pm$37.6ng (94.7%) in the 100ng sample and 122.1 $\pm$20.8ng (122%) when reanalysed one week later, when the 60ng

TABLE 12

UPTAKE INTO CELLS OF PLATINUM FROM CISPLATINUM AFTER 24 HOUR INCUBATION

Dose of Cisplatinum	PE/01	PE/C4
1.0uM	0, 3.9 (but AAS readings near background)	4.6, 4.9
5.0uM	7.2, 7.3	16.5, 21.6
(a)	21.4, 10.2	8.4, 22.4
10.0uM	55.8, 37.2	33.6, 41.9

(a) from the two time course experiments shown in figure 38.

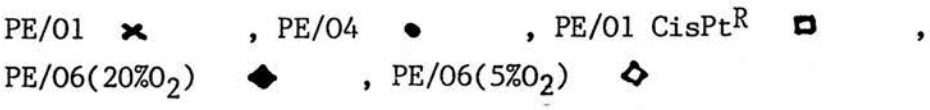
sample showed 72.0 ±9.9ng (120%) platinum detectable. These standards show the technique was reasonably accurate and serve to illustrate the variability observed.

In this third experiment PE/01 showed less cellular platinum than PE/04 after 24 hour incubation with 5uM cisplatinum but a similar level with 10uM cisplatinum incubation. However combining the results for 24 hour 5uM cisplatinum incubations from the 3 experiments gives 11.5 ±3.4 (S.E.) fgm platinum/cell for PE/01 and 17.2 ±3.2 (S.E.) fgm platinum/cell for PE/04 and these figures are not significantly different.

4.2.2 Results - [^{195m}Pt] cisplatinum uptake

In the first experiment cells from the cell lines PE/01, PE/04, PE/01 CisPt^{R}, PE/06 derived in 20% O_2, and PE/06 derived in 5% O_2 were incubated with 0.6, 2, 5 and 10ug/ml [^{195m}Pt] cisplatinum (2, 6.7, 16.7 and 33uM) for 70 minutes before the cells were spun down, washed and counted. [^{195m}Pt] cisplatinum standards at 0.5, 2.5, 5, 10 and 25ng/ml cisplatinum in PBS were counted at the same time. The results are shown in Figure 39. The amount of cellular platinum observed increased linearly with cisplatinum concentration in the incubation. By linear regression analysis the only significant difference in the slopes of the lines was for PE/06 (5% O_2) which had a significantly greater slope than PE/01 ($p = 0.02$), PE/01

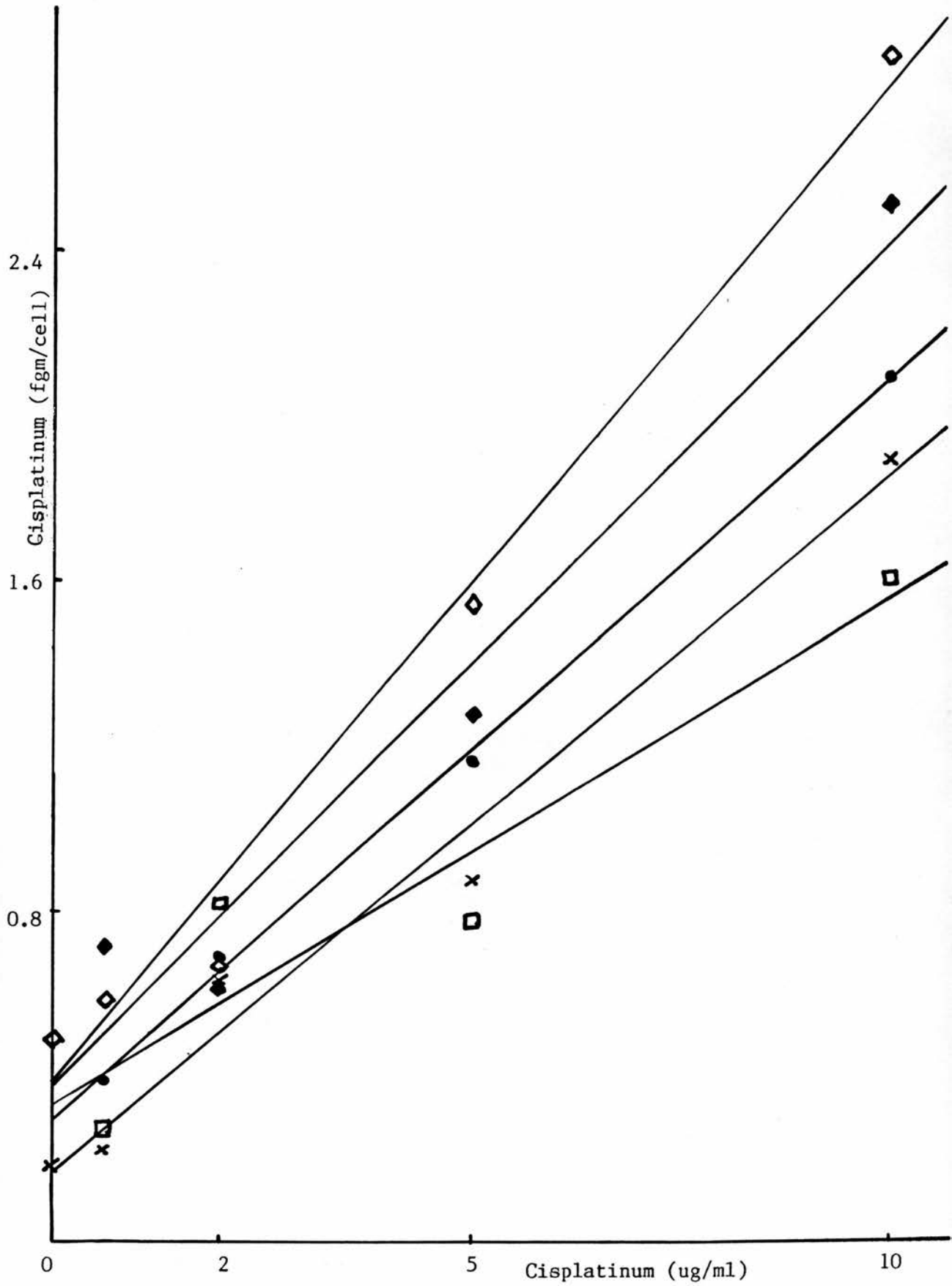
Figure 39 [^{195m}Pt] Cisplatinum Uptake after 1 hour



Cells were harvested from monolayer culture and incubated in suspension in RPMI-1640 with various concentrations of [^{195m}Pt] cisplatinum for 70 minutes and the cellular uptake of the radionuclide ^{195m}Pt then determined.

Linear regression lines are shown for each cell line calculated from the uptake at each dose of cisplatinum.

Figure 39



CisPt^R ($p = 0.02$) and PE/04 ($P = 0.05$). For the 4 lines grown in 5% CO_2/air there was no significant difference in the slopes implying no significant difference in uptake of cisplatinum into the cells after approximately 1 hour incubation with the drug. Even the difference between the PE/01 CisPt^R and PE/06 (5% O_2) lines at 10ug/ml cisplatinum is only 1.8 fold.

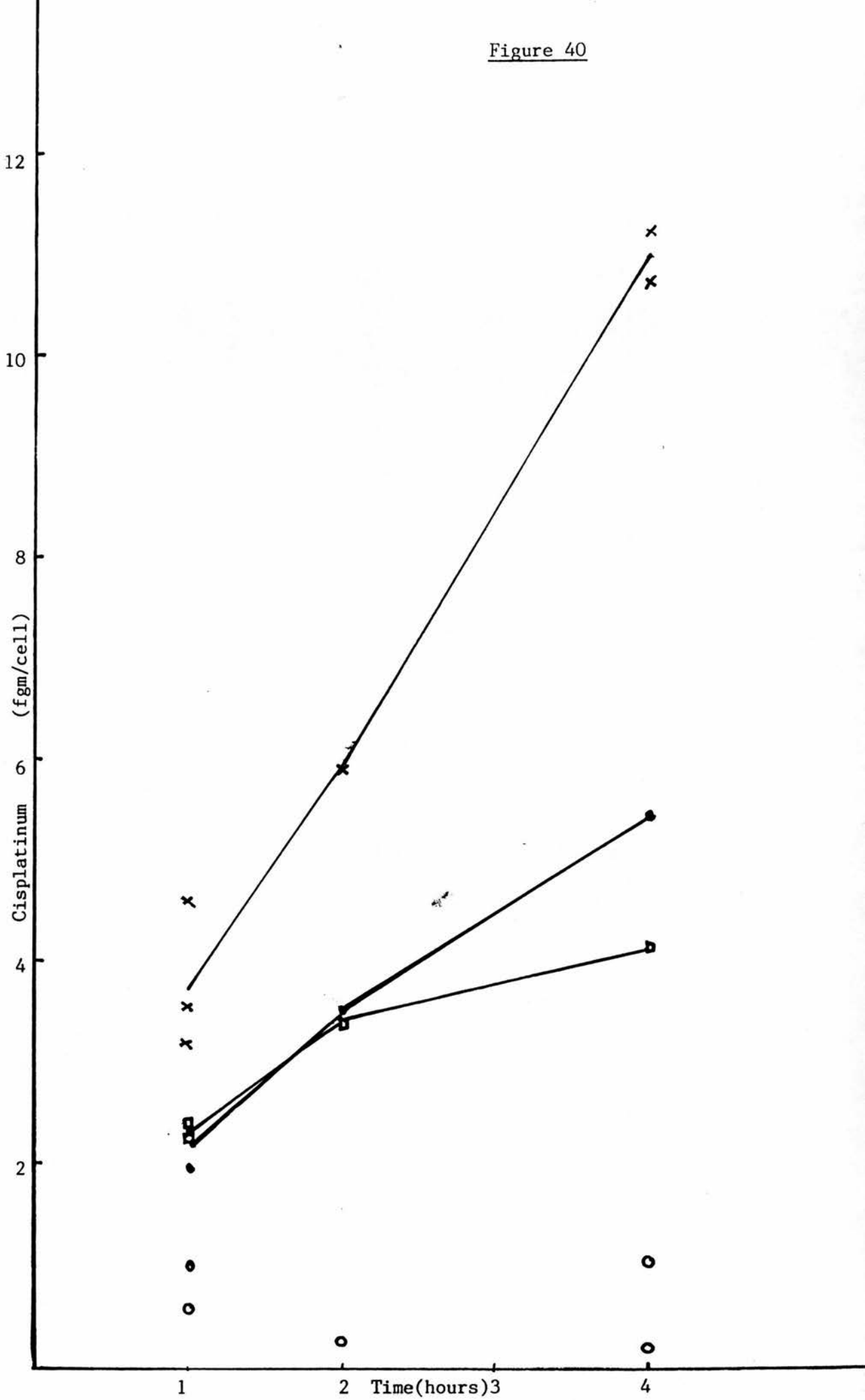
A second experiment was possible before the radionuclide had decayed too far. Cells of the PE/01, PE/04 and PE/01 CisPt^R lines were incubated with 10ug/ml [^{195m}Pt] cisplatinum (33uM) for 1, 2 and 4 hours before being spun down, washed and counted. Counts from uptake into 3×10^6 cells in 1 sample for each cell line at 1 hour instead of 5×10^6 cells showed similar values when converted to counts per cell. A recount after a third washing in PBS showed a small decrease in the counts and results as fgm cisplatinum/cell derived from these counts are shown in Figure 40. The uptake at 1 hour was slightly higher than in the previous experiment for PE/01 (3.2 versus 1.7 fgm cisplatinum/cell after minus background in controls) but gave similar values for the other two cell lines (PE/04 1.7 versus 1.8 fgm/cell; PE/01 CisPt^R 1.7 versus 1.2 fgm/cell). Uptake at 2 and 4 hours was higher in PE/01 than the other two resistant cell lines PE/04 and PE/01 CisPt^R.

Figure 40 Time Course of [^{195m}Pt] cisplatinum uptake

Cells were harvested from monolayer culture and incubated in suspension inRPMI-1640 for 1, 2, or 4 hours with 10 ug/ml [^{195m}Pt] cisplatinum before the amount of cellular ^{195m}Pt radionuclide was determined.

Multiple values at one time point represent duplicates. Background points at each time are shown and were for PE/01 cells incubated without radionuclide except for the higher of the 2 background points at 4 hours which used PE/01 CisPt^{R} cells.

Figure 40



All the above readings are on a per cell basis. The cell volumes of PE/01 and PE/01 CisPt^R are the same and PE/04 had a mean volume 77% of the volume of PE/01 but with the spread of cell volumes in each cell population, PE/04 was not significantly different to PE/01 in this respect (data in chapter 2). Thus the calculations here have not been adjusted for cell volume.

4.3 Discussion

In this chapter I report experiments attempting to measure platinum taken up into cells following cisplatinum exposure at doses as near as practicable to the biologically effective doses. However due to the relatively poor sensitivity of the available techniques the concentrations necessary to examine uptake of cisplatinum into cells (2-33uM) are still higher than the LD_{50s} seen in chapter 3 (0.23uM and 3.3uM for continuous or 1 hour dose of cisplatinum on PE/04).

Some of the data presented here suggests a difference in the amount of cisplatinum taken up into PE/01 and PE/04 cells, the most convincing of which is the data in figure 40 when more cisplatinum was observed in PE/01 cells. However the rest of the data, particularly the 24 hour atomic absorption spectroscopy data and the 1 hour radionuclide data (figure 39) suggest no difference in cisplatinum uptake while the time course using atomic

absorption spectroscopy is rather inconclusive. There was little evidence for decreased uptake in PE/01 CisPt^R compared with the more sensitive PE/04 line. If the calculated values for PE/04 are adjusted to take account of the smaller mean cell volume the data in figure 40 might indicate a decrease in uptake from PE/01 to PE/04 to PE/01 CisPt^R as might be expected for resistance due to an uptake difference. However a similar adjustment in figure 39 would suggest increased platinum uptake in PE/04 compared with PE/01 (and also in the 24 hour atomic absorption data at 5uM cisplatinum). Given the range of cell sizes in the population of PE/01 or PE/04 (data in chapter 2, no significant difference between the cell lines) any adjustment for cell volume in the uptake figures would not improve the significance of any of the differences observed.

Although the two techniques are not directly comparable, since one was done on a growing cell monolayer at 37^oC and the other on trypsinised cells in suspension at room temperature and probably only measuring bound platinum, the amounts of cellular platinum observed were of the same order of magnitude. 10uM cisplatinum for 24 hours gave approximately 40 fgm platinum equivalent/cell by atomic absorption spectroscopy or 0.2 fmoles/cell. 10ug/ml cisplatinum (33uM) for 4 hours gave 5-10 fgm cisplatinum/cell by radionuclide analysis or approximately

0.02-0.03 fmoles/cell.

The most detailed work on cisplatinum uptake has been down by Scanlon and co-workers (K.J. Scanlon et al, 1983; R.B. Gross et al, 1986; S. Shionaya et al, 1986) using the [^{195m}Pt] platinum isotope and they have emphasised the effect of cisplatinum on amino acid transport. In their initial study cisplatinum uptake was measured in L1210 cells after 1 hour at concentrations between 10 and 250uM cisplatinum in a Cross and Taggart buffer at 37^{o}C, with drug removed at the end by dilution and washing in ice-cold saline solution. Uptake was approximately 0.4, 2.4 and 4.9 fmoles/cell (values estimated from graph) at 10, 25 and 50uM cisplatinum in the incubation. The later more detailed work using L1210 and a subline resistant to cisplatinum (R.B. Gross et al, 1986) showed identical uptake over 10 minutes at 2 and 10uM cisplatinum, and similar uptake in the 2 lines over 75 minutes at 25uM cisplatinum in Earls salt solution (0.022 and 0.015 fmole/cell respectively) although they noted 44% less initial (background) binding of cisplatinum in the resistant cells after 10 seconds. They also observed virtually immediate efflux of approximately 50% of the platinum when the cells were diluted into drug free medium after 15, 30 or 60 minutes, although the absolute amounts of platinum uptake and that which remained bound had increased with time. Similar results were also observed

for K562 human chronic myelogenous leukemia cells and a subline resistant to cisplatinum (S. Shionaya et al, 1986). Incubation with 25uM cisplatinum for 75 minutes gave 0.022 and 0.019 fmole platinum/cell. Experiments done at 24°C instead of 37°C showed decreased uptake at about 0.010 fmole/cell although almost 50% could still efflux from the cell at this temperature. Thus my values using the radionuclide probably more nearly represent bound platinum (since the cells were washed at room temperature) and the values from the atomic absorption spectroscopy total cellular platinum. This question remains somewhat equivocal in any study where potential efflux during sample processing is not monitored. Subcellular localization studies on homogenized rat tissues (D.D. Choie et al, 1980; R.P. Sharma et al, 1983) show some 40% of the platinum as low molecular weight species in the final supernatant, designated cytosol, after separation of the cellular organelles.

Other workers using atomic absorption spectroscopy have shown similar values to those above for amount of platinum per cell but usually only bound platinum is represented. Uozumi reported approximately 0.018 up to 0.064 fmole platinum/cell in FM3A mouse tumour cells after a 30 minute incubation with from 40 to 160uM cisplatinum (J. Uozumi et al, 1984) and Ciccarelli reported approximately 0.003, 0.014 and 0.025 fmole/cell for CV-1 African green monkey

kidney cells after a 48 hour incubation with 1, 5 and 10uM cisplatinum (R.B. Ciccarelli et al, 1985).

However variations between different cell types have been noted. Eichholtz-Wirth, using proton induced characteristic x-ray emission analysis, observed HeLa cells to show 1.3 fold more cellular platinum than chinese hamster lung fibroblast cells and 4.4 fold more than syrian hamster kidney cells (H. Eichholtz-Wirth et al, 1986) which correlated well with their sensitivity to cisplatinum. Another recent study using two human prostate carcinoma cell lines also showed a difference in uptake of cisplatinum (using the ^{191}Pt radioisotope) correlated with differential sensitivity to the drug and further correlated this with the amount of platinum bound to DNA and protein (S.A. Metcalfe et al, 1986). Other preliminary reports, reviewed recently by Fry, mainly using L1210 cells and resistant sublines, also suggest some changes in cisplatinum uptake or amount of cellular bound platinum (D.W. Fry et al, 1986) correlated with drug resistance.

One problem with all these studies has been the large numbers of cells and high cisplatinum concentrations which need to be used given the sensitivity of the techniques to measure platinum (and in my study here I noticed an increased matrix interference on the atomic absorption

signal from the platinum with increased cell number) and the poor suitability and availability of platinum radioisotopes. Too high a dose of cisplatinum can give results of doubtful biological relevance. This accounts for the small amount of uptake data for cisplatinum relative to many other drugs. One recent preliminary report has used the close cisplatinum analogue ethylenediamine dichloro platinum (II) labelled with ^{14}C in the stable ethylenediamine ligand (W.R. Waud et al, 1985) and this may be an easier and more sensitive method of examining uptake and bound drug provided the cell sensitivity to this analogue parallels that of cisplatinum. Nevertheless the above reports indicate that uptake of cisplatinum or other membrane effects may be important for resistance to the drug in some systems. My data reported here are not strong enough to conclude that there is an uptake difference in the PE/01 family of ovarian carcinoma cell lines which would be significant to explain the resistance observed.

5. Mechanisms of resistance to cisplatinum - Glutathione studies

Since the early 1960s thiols, compounds with free SH groups, have been implicated in resistance to alkylating agents (I. Hirono, 1961; T.A. Connors, 1966). Glutathione is the principal non-protein thiol in cells and its metabolism has been recently reviewed (A. Meister et al, 1983). Its importance in protecting cells against oxidative stress and radiation has received wide attention (J.E. Biaglow et al, 1983; J.D. Chapman et al, 1984) but it is thought to play a role in the detoxification of a variety of drugs, not just alkylating agents (B.A. Arrick et al, 1984). The group of enzymes associated with glutathione metabolism is obviously important and the selective modification of their activity has been recently reported (A. Meister, 1983). Amongst these enzymes the importance of glutathione transferases in reacting glutathione with electrophilic drugs has been recognised (L.F. Chasseaud, 1979). The depletion of intracellular glutathione can be achieved by inhibiting the enzyme γ-glutamylcysteine synthetase, the penultimate step in glutathione synthesis, with the specific inhibitor buthionine-S, R-sulfoximine (BSO) (C.W. Griffith et al, 1979) and this has proved much more selective than using thiol reactive agents like diethyl maleate to deplete glutathione levels. Since the work of Griffith a number of authors have reported the use of BSO to demonstrate

various glutathione effects. In L1210 cells resistant to melphalan where detoxification by glutathione had been emphasised as the important mechanism of resistance (K. Suzukake et al, 1983), sensitivity to melphalan could be restored by reduction in cellular glutathione by buthionine sulfoximine (S. Somfai-Relle et al, 1984). A similar potentiation of melphalan toxicity by glutathione depletion has also been observed in 3 human ovarian carcinoma cell lines and a sub-line made resistant to melphalan in vitro (J.A. Green et al, 1984).

Thiol compounds also protect against cisplatinum toxicity and have been used to reduce its nephrotoxicity. In rats sodium diethyldithiocarbamate could be administered to protect the kidney but to have minimal effects on the toxicity of cisplatinum against a subcutaneous tumour (R.F. Borch et al, 1980). It can also reduce bone marrow toxicity (R.G. Evans et al, 1984) and has been shown to inhibit and reverse the reaction of cisplatinum with proteins in vitro (S.L. Gonias et al, 1984). In a similar way sodium thiosulphate reduces nephrotoxicity in mice (S.B. Howell et al, 1980) and can thus improve the therapeutic ratio of cisplatinum (Y. Iwamoto et al, 1984). Recently I.V. thiosulphate has been used to protect against the systemic toxicity of i.p. cisplatinum in humans (S.B. Howell et al, 1983). Thiosulphate appears to bind to cisplatinum and thus prevent its uptake into cells

in vitro (J. Uozumi et al, 1984). Treatment with glutathione itself has also shown protective effects in vivo (F. Zunino et al, 1983). Litterst and his colleagues have shown changes in glutathione and glutathione-S-transferase levels in rat liver and kidney after cisplatinum treatment and suggested an association of cisplatinum with glutathione in the tissue cytosols (C.L. Litterst et al, 1982).

In this laboratory preliminary data by Dr. Adams had shown differences in glutathione content and levels of glutathione-S-transferases between the PE/01 and PE/04 cell lines (C.R. Wolf et al, 1984 abstract) with glutathione concentrations of 0.8 and 5.9 nmol/10^6 cells and transferase activities 35 and 159 nmol CDNB conjugate/min/10^6 cells for PE/01 and PE/04 cells respectively in logarithmic phase cultures. Further work by Lewis (ICRF Laboratory of Molecular Pharmacology and Drug Metabolism, Edinburgh) has confirmed this difference at about 2-fold for both glutathione and glutathione-S-transferase activity. To see whether this difference is important to cisplatinum resistance I have measured glutathione levels in the cell lines and the resistant subline PE/01 $CisPt^R$, and attempted to restore sensitivity to cisplatinum using the glutathione synthesis inhibitor BSO in an analogous manner to that of Green (J.A. Green et al, 1984).

5.1 Methods

BSO toxicity on PE/01 and PE/04 and the effect of cisplatinum plus BSO on PE/04 was assessed at low cell density by the same clonogenic assay on plastic used in chapter 3. Two days after being plated out the cells were treated with 50 or 100uM BSO for 19 hours and then treated with cisplatinum on the 3rd day for $2\text{-}2^1/_2$ hours with BSO still present before the medium was changed to fresh medium without drugs. Alternatively cultures in $25cm^2$ or $75cm^2$ flasks (Nunc Gibco Ltd) at higher cell density but in logarithmic phase were treated with up to 1mM BSO or cisplatinum plus 1mM BSO, harvested immediately by trypsinisation and plated out at low cell density into 35mm wells of 6-well plates in triplicate in the clonogenic assay on plastic as above. All incubations were at 37°C in a 5% CO_2/air high humidity incubator.

Measurement of reduced glutatione (GSH) levels was by the fluorometric assay of Mokrasch (L.C. Mokrasch et al, 1984) with some modifications. 5% trichloroacetic acid was used as a protein precipitant instead of formic acid since formic acid did not remove all the protein from the cell supernatant (no visible precipitate versus considerable precipitate from 5% TCA). In my attempts to use the assay to quantitate lower levels of glutathione I found a standard additions method gave more accurate results than reading values from a standard curve since although a good

linear increase in glutathione measured with increasing cell number was observed the line did not extrapolate through zero as shown in figure 41.

Between 1.5 and 8 x 10^6 cells growing in $25cm^2$ or $75cm^2$ flasks were harvested by trypsinisation into cold phosphate buffered saline. A small portion was removed for later cell counting and the rest were spun down, resuspended in 1ml cold PBS and transfered to a microfuge tube and then repelletted and resuspended in either 500ul or 1ml of cold 5% TCA. Reduced glutathione (GSH) in solution is stable for a reasonable length of time when kept cold and especially so in acidic conditions to prevent its oxidation so that this initial part of the procedure was done as quickly as possible. The cells in cold 5% TCA were then sonicated (3 X 5 second pulses with 10 second intervals in iced water to prevent the solution warming up) and the precipitate spun down in a microfuge (20,000g for 20 minutes at 4^oC). A sample of the supernatant was taken for glutathione determination.

A 10ul sample of the supernatant was mixed with 10ul of buffered formaldehyde (1:4 (v/v) 37% formalin : 0.1M Na_2HPO_4). After 1 to 2 minutes 10uls of a reduced glutathione standard solution in buffer (0.1M Na_2HPO_4, 5mM EDTA, pH 8.0) was added followed by 2mls of this buffer and then 100uls of o-phthalaldehyde (1 mg/ml in methanol).

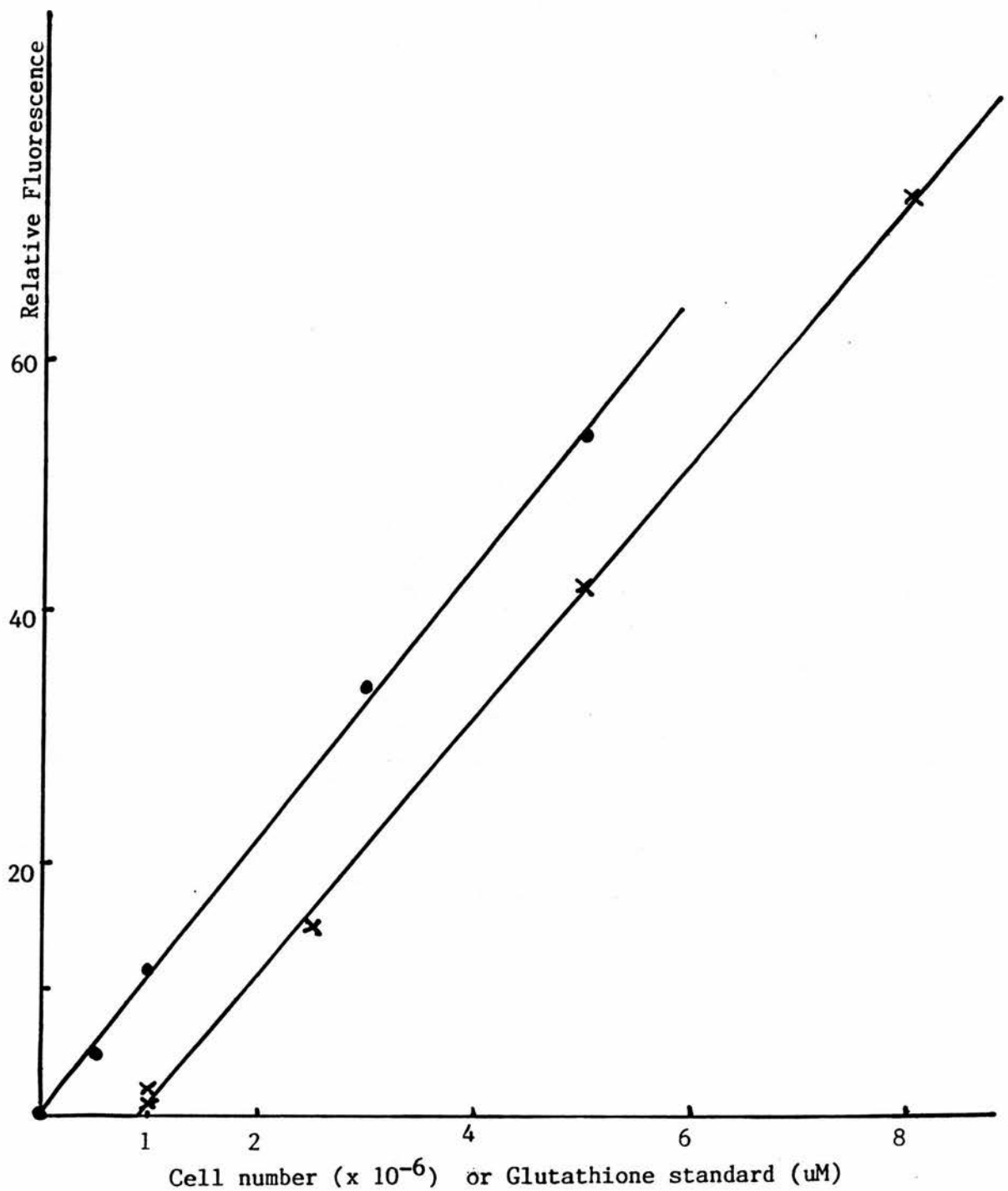
Figure 41 Fluorometric assay for Glutathione - with increasing cell number

PE/O1 cells ×
Glutathione standards ●

PE/O1 cells were harvested and various cell numbers resuspended in 1ml of 5% TCA. A 100ul aliquot of the cell supernatant was used to measure reduced glutathione levels.

A standard curve of various concentrations of reduced glutathione in 5% TCA is shown for reference.

Figure 41


Relative Fluorescence
60
40
20
1
2
4
6
8
Cell number (x 10^{-6}) or Glutathione standard (uM)

This final assay mix was left in the dark at room temperature for 35-60 minutes before reading the fluorescence on a Baird Nova spectrofluorimeter (excitation wavelength 345nm and emission wavelength 425nm). Reduced glutathione standards and o-phthalaldehyde solutions were made up fresh daily. Three glutathione standards between 2uM and 50uM final concentration were usually used.

In some assays 100ul samples of the supernatant plus glutathione standard in 5% TCA were used with 100ul of buffered formaldehyde and 1ml of buffer (solution volumes as in the assay of Mokrasch) in an attempt to assay relatively low cell numbers. Although similar results to the above method were obtained, drifting and variation of the fluorescence reading were observed. This was due to the low pH (approximately pH7.1) in the final reaction volume due to using trichloroacetic acid instead of formic acid. Hissin had previously reported the importance of the pH for the reaction with o-phthalaldehyde with the fluorescence intensity decreasing below pH 8.0 (P.J. Hissin et al, 1976). With a 10ul sample of TCA and 2mls of buffer a pH of 8.0 was maintained. In this study oxidized glutathione (GSSG) was not measured and it represents only a minor proportion of the total glutathione (A.D. Lewis, unpublished results; A. Meister et al, 1983).

5.2 Results

BSO toxicity

PE/01 and PE/04 cells were incubated with BSO for approximately 22 hours in the clonogenic assay to ascertain a minimally toxic dose to use in the BSO + cisplatinum experiments. The results are shown in figure 42, combined from experiments with incubations between 18 and 25 hours with BSO. A preliminary experiment with a 47 hour incubation showed a similar result but with toxicity at lower concentrations of BSO. PE/01 was much more sensitive than PE/04 and also showed sensitivity at levels of BSO (35% survival at 20uM BSO for 22 hours, 8% survival at 10uM BSO for 47 hours) less than those used for melphalan potentiation by Green (J.A. Green et al, 1984) or doses used in later work from the same group at the NCI (K.G. Louie et al, 1985). Thus further work with BSO was confined to PE/04.

In 3 separate later experiments with logarithmic phase PE/04 cells growing in $25cm^2$ or $75cm^2$ flasks no toxicity was observed with concentrations up to 1mM BSO for 22 hours. Clearly the change in the condition of the cells, perhaps the higher cell density, helped them maintain viability at concentrations which were toxic in the usual assay.

Figure 42 BSO Toxicity on cells at low cell density

PE/01 ×——×

PE/04 ●- - -●

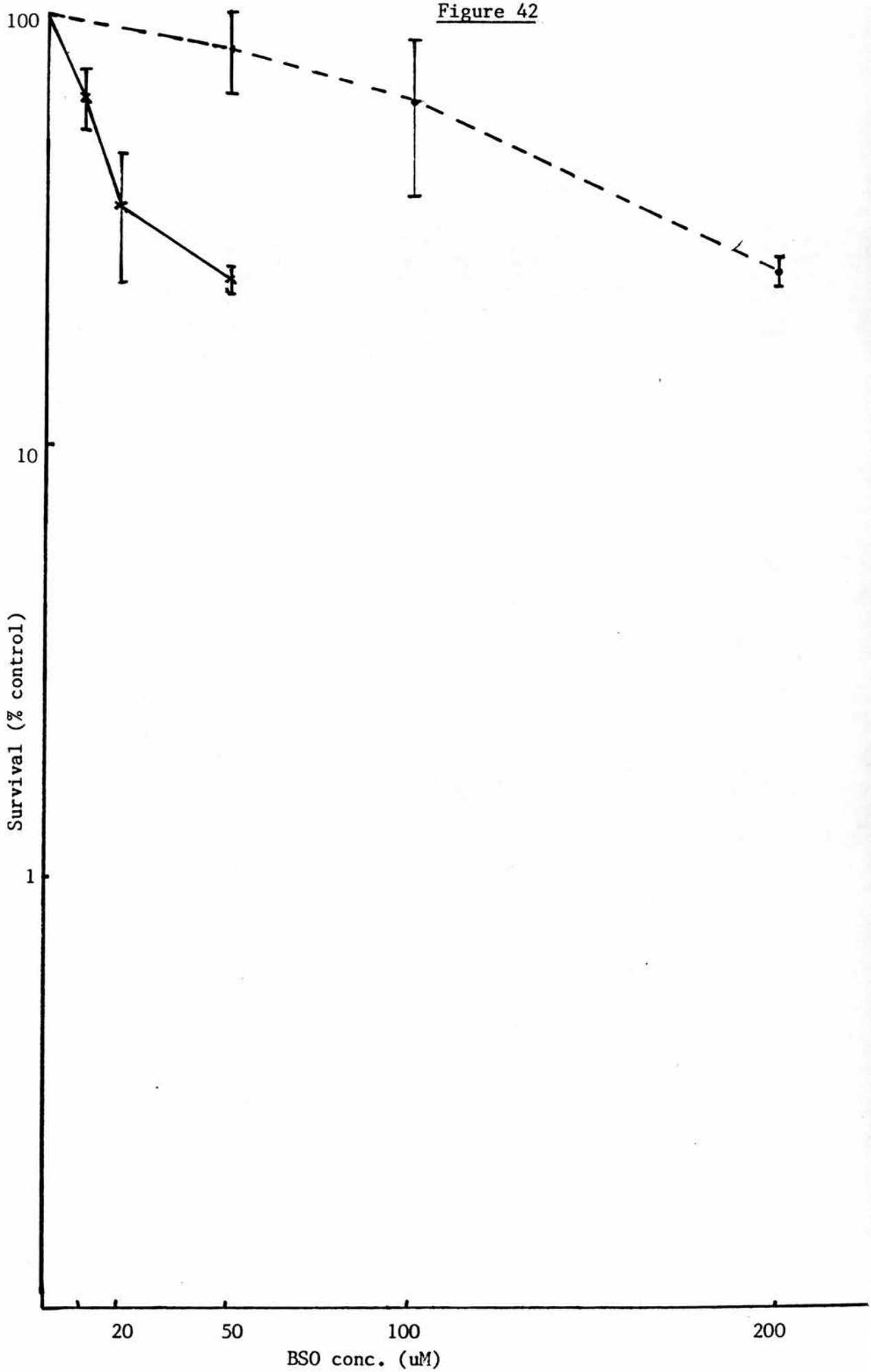
BSO dose[a] (uM)	% Survival PE/01	PE/04
10	63.6 ± 10.1(2)[b] (61.0)(c)	-
20	35.6 ± 11.8(3) (32.8)	146.7 (1) (105.7)
50	24.1 ± 1.7 (3) (24.0)	82.7 ± 17.0(5) (75.6)
100	28.5 (1) (32.2)	62.3 ± 24.4(5) (46.9)
200	-	25.3 ± 2.2 (3) (43.0)

(a) exposure for 18-25 hours in different experiments.

(b) BSO toxicity assessed by the clonogenic assay on plastic reported in chapter 3. Results using the [^{3}H]-thymidine method for calculating clonogenic survival after drug exposure are shown.
The number of experiments from which the mean and standard error were calculated is shown in brackets.

(c) Mean survival calculated from visual colony count of 1 well per triplicate is shown in brackets.

Figure 42



Cisplatinum + BSO toxicity

In the first experiment PE/04 cells (at low density in 6 well plates) were pretreated with 50uM BSO before a range of cisplatinum doses as shown in Figure 43. BSO alone reduced survival to 76% of the control and the Cisplatinum plus BSO line has been adjusted to 100% to see if there was any potentiation of cisplatinum toxicity. Some difference is apparent in the lines using the [^{3}H]-thymidine pulse method of colony number assessment at the end of the experiment although the slopes of the lines are similar. However the visual colony count of 1 well per triplicate, which showed reduced survival to 82% of control by BSO alone, after adjustment for this effect showed no potentiation of cisplatinum toxicity except at the highest dose.

In a second experiment repeated in the same manner the dose of BSO was increased to 100uM and the results are shown in Figure 44. Here the BSO alone wells were 112% of control. After adjustment to 100% there was some small decrease in survival with cisplatinum plus BSO but the slope of the drug survival curves were again very similar suggesting BSO had little potentiating effect.

After BSO at concentrations up to 1mM had been shown to be non-toxic to PE/04 in bulk culture, logarithmic phase PE/04 cells in 75cm^2 culture flasks were treated with 1mM

Figure 43 PE/04 - Cisplatinum toxicity
± BSO (50uM)

Cisplatinum alone ●——●

Cisplatinum with BSO pretreatment o——o

Cells were treated in the clonogenic assay on plastic with 50 uM BSO for 19 hours before exposure to cisplatinum for 2 hours with BSO still present.
Mean and standard error from triplicates in one experiment using the [^{3}H]-thymidine method to calculate clonogenic survival.
BSO alone reduced survival to 76% - adjusted to 100% in the graph for comparison with the cisplatinum toxicity without BSO treatment.

The clonogenic survival calculated from a visual colony count of 1 well per triplicate is also shown.
BSO alone reduced survival to 82% - adjusted to 100% in the graph for comparison.

Cisplatinum alone ●- - -●

Cisplatinum with BSO pretreatment o- - -o

Figure 43

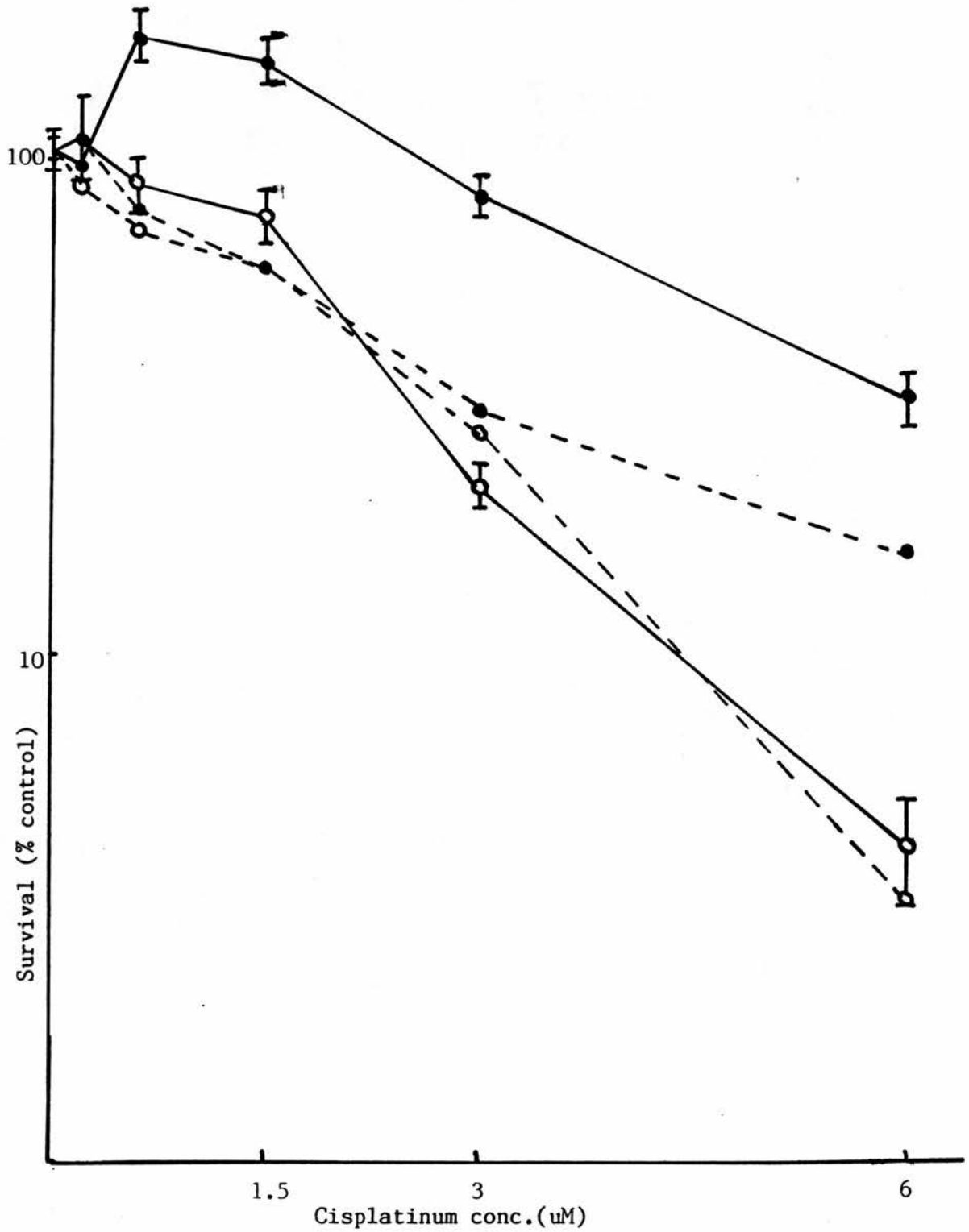
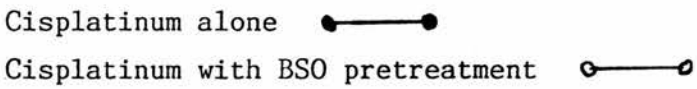
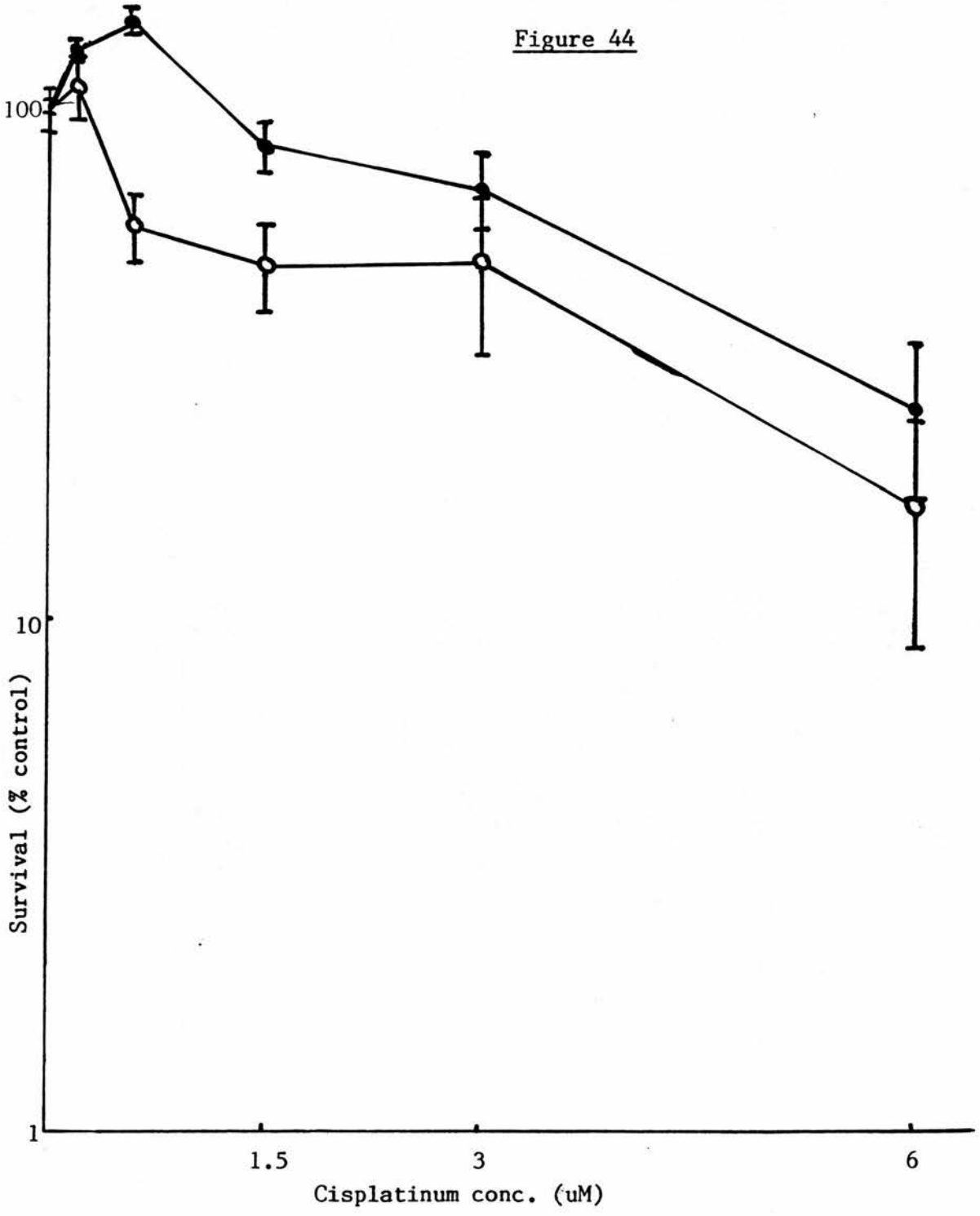


Figure 44 PE/04 - Cisplatinum toxicity
± BSO (100uM)



Cells were treated in the clonogenic assay on plastic with 100 uM BSO for 19 hours before exposure to cisplatinum for $2^1/_2$ hours with BSO still present. Mean and standard error from triplicates in one experiment using the [^{3}H]-thymidine method to calculate clonogenic survival.
BSO alone gave 112% survival - adjusted to 100% in the graph for comparison with the cisplatinum toxicity without BSO treatment.

Figure 44



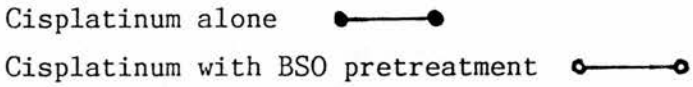
BSO for 22 hours and then treated with various concentrations of cisplatinum for 2 hours in the presence of BSO. After plating out to low cell density for the clonogenic assay, no difference in the cisplatinum survival curves with or without BSO was observed as shown in Figure 45. It could also be seen that the cisplatinum toxicity was the same in this bulk culture as it was at low cell density in Figure 43 and 44.

Glutathione concentrations

The mean reduced glutathione concentration for PE/04 cells in logarithmic phase in 3 experiments was 5.40 $\pm$ 2.38 (SEM) nmoles GSH/10^6 cells. Different BSO concentrations from 50uM to 1mM depleted the cellular glutathione to similar levels (12.7% for 50uM BSO and 6.8% for 1mM BSO in one experiment and less than 10% of control for all BSO concentrations from 50uM to 1mM in a second experiment) which suggested that 50uM BSO should be sufficient to deplete the glutathione for the potentiation studies above. Glutathione concentrations for PE/01 and PE/01 $CisPt^R$ in logarithmic cultures were 1.24 and 1.95 nmoles GSH/10^6 cells (in 1 experiment where PE/04 gave a value of 3.20 nmoles/10^6 cells).

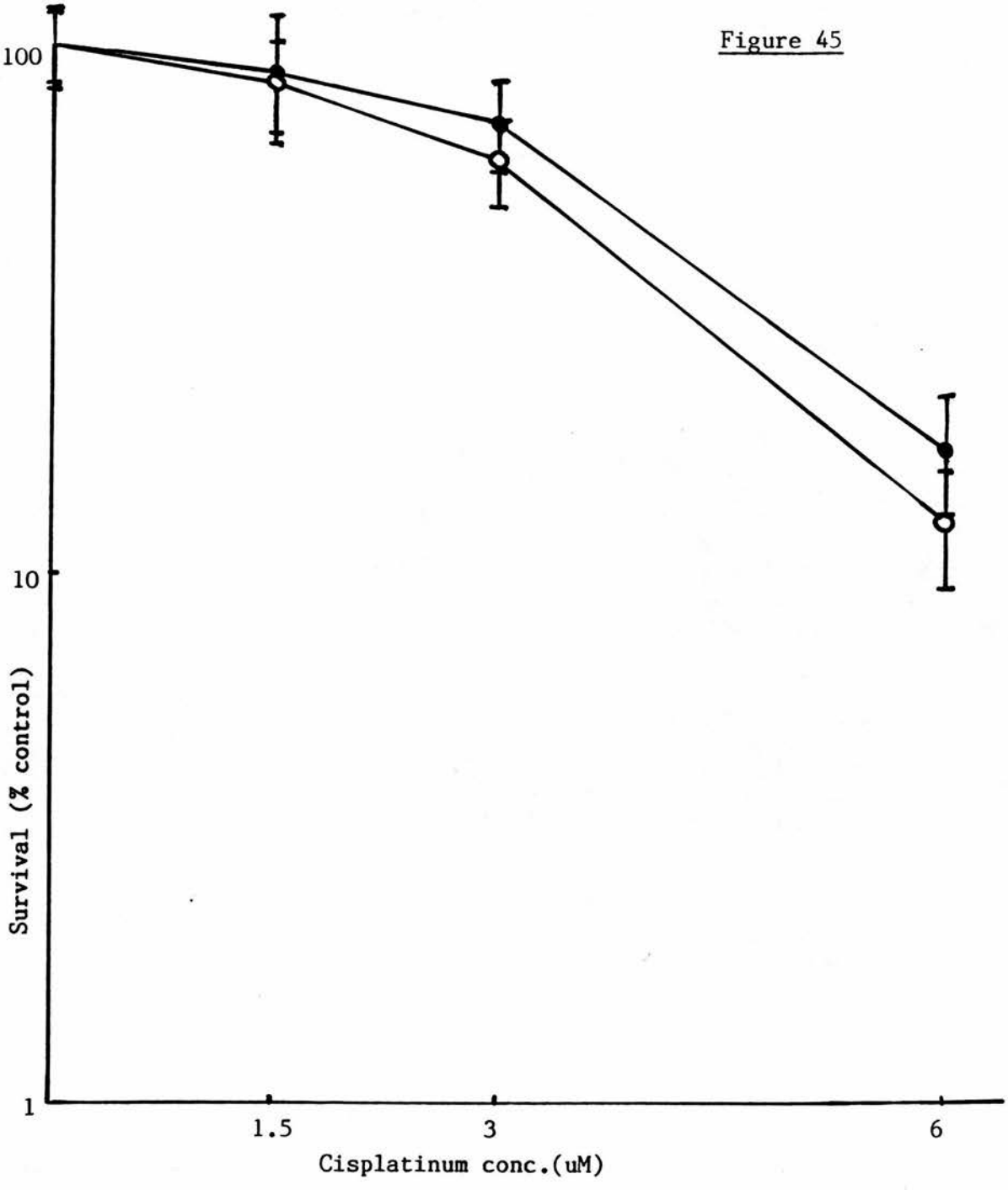
Assays using 100ul of cell supernatant leading to a lower pH in the final fluorescence reaction solution gave similar values for the glutathione concentrations. Mean

Figure 45 PE/04 - Cisplatinum toxicity ± BSO (1mM)



PE/04 cells in logarithmic phase of growth (2 days after trypsinisation of 2.5×10^6 cells into 75 cm^2 flasks) were treated with 1mM BSO for 22 hours followed by cisplatinum for 2 hours with BSO still present.
Cells were harvested immediately and plated out at low cell density in fresh medium in the clonogenic assay on plastic.
Mean and standard error are from triplicates in the one experiment using the [^{3}H]-thymidine method to calculate clonogenic survival.
BSO alone gave 99% survival - adjusted to 100% in the graph for comparison with the cisplatinum toxicity without BSO treatment.

Figure 45



values including these experiments are shown in Table 13 for PE/01, PE/04 and PE/01 CisPt^R. PE/06 (20%O_2) and PE/06(5%O_2) gave values of 4.7 and 9.6nmoles GSH/10^6 cells in one experiment. A confluent culture of PE/01 gave a value of 0.3nmoles GSH/10^6 cells in one experiment.

The GSH concentrations obtained here for PE/01 and PE/04 are similar to those obtained using an HPLC assay (paper shown in appendix, C.R. Wolf et al, submitted for publication 1986) and decreased glutathione levels in confluent versus logarithmic cultures have also been shown (A.D. Lewis et al, 1986). In this work I tried to use only cells in logarithmic culture 2 to 3 days after passaging. Glutathione content has been calculated as nmol/10^6 cells as reported by others and not adjusted for cell volume differences between the cells which were minimal (reported in Chapter 2 - as a percentage of the PE/01 mean cell volume, the cell volume of the PE/04 and PE/01 CisPt^R lines was 77% and 97% respectively but all three cell lines showed a range of cell size with no significant difference in cell volume between them).

5.3 Discussion

This work has confirmed the difference in reduced glutathione levels between PE/01 and PE/04 and in addition shown that PE/01 CisPt^R has slightly elevated GSH levels compared with its parent PE/01 although not greater than

TABLE 13
CELLULAR REDUCED GLUTATHIONE CONCENTRATIONS

	nmoles GSH/10^6 cells[a]
PE/01	1.97± 0.46
PE/04	4.45± 1.10[b]
PE/01 $CisPt^R$	3.11 ± 0.64[c]

(a) values are mean (and standard error) of data from 4, 7 and 3 experiments for PE/01, PE/04 and PE/01 $CisPt^R$ in logarithmic phase cultures.

(b) significantly different from PE/01 ($P<0.01$).

(c) significantly different from PE/01 but not from PE/04 (at $p = 0.05$).

PE/04. Changes in glutathione levels with tissue culture conditions, time since passaging and position in the cell cycle (J.K. Dethmers et al, 1981; G.B. Post et al, 1983; L. Roizin-Towle, 1985) have been reported by others so some variability might be expected in different experiments but I attempted to minimise this by only using good growing cultures in logarithmic phase.

Minimal potentiation of cisplatinum toxicity was observed with BSO treatment where glutathione concentrations were reduced to less than 10% in PE/04 cells. Other workers have used higher levels of BSO than initially used in this project (1mM BSO, J.K. Dethmers et al, 1981; 2mM BSO, B.A. Arrick et al, 1982; 0.5mM BSO with ovarian carcinoma cell lines, P.A. Andrews et al, 1985) for a similar length of time without toxicity and levels up to 10mM BSO have been used for shorter times (A. Russo et al, 1984). However the glutathione level and extent of depletion by BSO reported here is similar to that reported in the literature (see references below). The residual 5-10% glutathione is reported to resist depletion (E.C. Gaetjens et al, 1984) or its loss be associated with marked BSO toxicity (J.K. Dethmers et al, 1981). The greater sensitivity to BSO toxicity at low cell density would suggest even greater glutathione depletion under these conditions as has been shown with glutathione depletion by spermine (A. Russo et al, 1985). Even at this low cell density little

potentiation was evident.

These results confirm those of Andrews with ovarian cell lines and sublines who could potentiate melphalan and chlorambucil (and interestingly trans-platinum) but not cisplatinum toxicity (P.A. Andrews et al, 1985) and a preliminary report by Holden using a head and neck squamous cell carcinoma cell line made resistant to cisplatinum (S.A. Holden et al, 1985). Andrews also found no increase in glutathione levels in the sublines with resistance acquired in vitro to cisplatinum although glutathione levels could be increased 2-3 fold in sublines made resistant to melphalan. However a recent preliminary report by Andrews with more highly resistant sublines (9-13 fold resistant instead of 2-3 fold resistant) showed increased glutathione levels and the resistance could be partially reversed (to about 4-fold) by BSO treatment (P.A. Andrews et al, 1986). In my work here the PE/01 $CisPt^R$ subline has 25-fold resistance with only a 1.6-fold increase in glutathione level with less glutathione than the PE/04 cell line. Further work from the NCI group using cisplatinum as well as melphalan did show potentiation of cisplatinum toxicity by BSO treatment but always to a lesser extent than for melphalan (dose modification factors approximately half, T.C. Hamilton et al, 1985). The different results obtained may be related to the extent of cross-resistance between cisplatinum and

melphalan in the different studies, with higher cross-resistance in the cell lines of the NCI group.

BSO has been thought to be a relatively non-toxic agent (S. Somfai-Relle et al, 1984) depleting glutathione with little effect on the rate of cell division (E.C. Gaetjens et al, 1984) and other cellular metabolism (B.A. Arrick et al, 1982) although others have regarded it as a cytostatic agent (R.F. Cordeiro et al, 1986). Gaetjens has adapted L1210 cells to proliferate continuously, with reduced glutathione content, in BSO concentrations as high as 10mM (E.C. Gaetjens et al, 1984).

The amount of glutathione depletion by BSO or enhancement in various cell lines is not linearly related to the expected toxicity of a drug like melphalan (T.C. Hamilton et al, 1985). A 2-fold increase in glutathione content does not necessarily indicate a 2-fold increase in melphalan resistance and observations vary widely in different systems. To further assess the importance of glutathione in drug metabolism it is also possible to increase intracellular glutathione levels with 2-oxothiazolidine-4-carboxylate (OTZ) which is converted intracellularly to cysteine by the enzyme 5-oxo-L-prolinase and thus stimulates glutathione synthesis (A. Meister, 1983). Glutathione levels 135-170% of controls led to modest protection against melphalan and cisplatinum

(1.2 and 1.4 fold) in human lung fibroblasts (A. Russo et al, 1986).

The precise mechanism of the protective effect of glutathione against these drugs is unknown. Although glutathione will covalently conjugate to many electrophilic agents often via a glutathione-S-transferase (L.F. Chasseaud, 1979) evidence for direct conjugation to melphalan or cisplatinum is equivocal (J. Levi et al, 1980; D.F. Long et al, 1981; C.L. Litterst et al, 1982). However the conversion of melphalan to its non-toxic derivative dihydroxy-melphalan has been related to the intracellular concentration of glutathione (K. Suzukake et al, 1983). A methionine substitution product of cisplatinum has been tentatively described (P.T. Daley-Yates et al, 1984). Since intracellular glutathione concentrations are in the millimolar range (data here; Meister et al, 1983), well in excess of drug concentrations, its depletion presumably affects the activity of some enzyme system, perhaps by being in the range of the Km of a glutathione transferase as discussed by Jakoby (W.B. Jakoby, 1978). Others have also suggested that glutathione may play just as critical a role in repair of drug induced damage (L. Roizin-Towle et al, 1984).

Significant changes in the levels of glutathione dependent

enzymes can also occur. Tew and his colleagues have shown an increase in glutathione-S-transferase activity and a decrease in glutathione reductase activity in a Walker 256 rat carcinoma cell line resistant to bifunctional nitrogen mustards and collaterally sensitive to nitrosoureas (A.L. Wang et al, 1985; K.D. Tew et al, 1985). PE/04 had increased glutathione-S-transferase activity over that of PE/01 (C.R. Wolf et al, 1984 abstract). More recent work has also shown increased activity of glutathione peroxidase, glutathione reductase and γ-glutamyl transpeptidase in PE/04 compared with PE/01 (A. Lewis et al, 1986 abstract). All these enzymes are potentially involved in drug detoxification.

Attempts to translate these results in vitro to in vivo systems have so far not met with much success. In one recent study, L1210 cells metastatic to the liver had a 2-fold increase in their glutathione content over that in cells in ascitic fluid of the peritoneal cavity (S. Ahmed et al, 1986). This effect, apparently due to the hepatic microenvironment, was accompanied by increased resistance to melphalan which negated the sensitization achieved by BSO treatment in the peritoneal cavity. In our laboratory others have shown altered glutathione levels in the bone marrow induced by a priming dose of cyclophosphamide where the time course of glutathione overshoot to increased levels paralleled the reduced toxicity of a subsequent

dose of cyclophosphamide (D.J. Adams et al, 1985). To improve the therapeutic index of treatment differential effects on host and tumour tissue will be necessary. These may be effected by differences in the timing of glutathione modulation in host and tumour.

The conclusion from the present work is that glutathione appeared to be of little importance to the drug resistance observed despite the differences in glutathione and GSH-related enzymes. A survey of the literature also indicates that glutathione is probably less important for protection against cisplatinum than against the bifunctional nitrogen mustards melphalan and chlorambucil. However since glutathione levels can change in different environments (S. Ahmed et al, 1986) and cisplatinum at high concentrations in vitro is known to inhibit γ-glutamyl transpeptidase (D.L. Bodenner et al, 1986) and other enzymes containing sulphydryl groups (J.L. Aull et al, 1979), some influence of metabolism at this level can not be entirely discounted. Obvious further experiments would involve attempted potentiation with BSO of melphalan and chlorambucil toxicity in these cell lines to see if there was a different effect to that with cisplatinum. Modulation of glutathione metabolism at low oxygen tension would also be worthwhile particularly in the light of the changed chlorambucil toxicity at low oxygen tension shown in Chapter 3 and the well studied role of glutathione in

hypoxic and aerobic cell responses to radiation (J.D. Chapman et al, 1984). It is also possible that the sulphydryl rich metallothionein protein may play a role in resistance to cisplatinum as shown in cell lines induced with cadmium (A. Bakka et al, 1981), although metallothionein is not thought to be induced by cisplatinum (A. Kraker et al, 1985; S. Kinsler et al, 1985) even though it is a normal component of many cells.

Obviously further investigations of drug resistance will need to be aware of variations in glutathione metabolism since it is an important general route of detoxification of electrophiles and at least monitor non-protein thiol levels and perhaps protein thiols as well, for example in the other ovarian cell lines described in chapter 2.

6. Mechanisms of resistance to Cisplatinum - DNA studies

The major target for cisplatinum toxicity is DNA (J.J. Roberts et al, 1979) and early studies established that DNA synthesis is inhibited before and at lower concentrations of drug than RNA or protein synthesis (H.C. Harder et al, 1970). As discussed in the introduction (Chapter 1), it remains uncertain as to which DNA lesions are critical to toxicity but most attention has concentrated on interstrand crosslinks and more recently intrastrand crosslinks. A number of recent reviews have considered the likely important DNA adducts (S.J. Lippard, 1982; B. Rosenberg 1985; A.L. Pinto et al, 1985; J. Reedijk et al, 1985). Altered DNA repair of these lesions has been postulated to account for cellular resistance to cisplatinum (M. Fox 1984).

Efforts to understand the toxicity of cisplatinum at the DNA level have basically proceeded in three directions. Firstly a number of authors have shown increased toxicity of cisplatinum towards cells deficient in DNA repair pathways in well characterised bacterial mutants (R. Alazard et al, 1982; M. Germanier et al, 1984; R.J. Fram et al, 1985; I. Husain et al, 1985; D.J. Beck et al, 1985) and yeast cells (M.A. Hannan et al, 1984), but also in human xeroderma pigmentosum and Fanconi anaemia mutants (H.N.A. Fraval et al, 1978; E.H.A. Poll et al, 1984). Secondly attention has focussed on what DNA adducts are

produced and in what frequencies by cisplatinum and its analogues (A. Eastman, 1985; A.M.J. Fichtinger-Schepman et al, 1985). It has recently become apparent that the most abundant adduct is an intrastrand crosslink between neighbouring guanine bases. This adduct has been quantitated immunochemically by two grcups (S.J. Lippard et al, 1983; A.M.J. Fichtinger-Schepman et al, 1985) and accounts for 35-45% of all adducts in chinese hamster ovary cells (A.C.M. Plooy et al, 1985). Thirdly, effort has concentrated around the technique of alkaline elution which has allowed sensitive analysis of DNA interstrand crosslinks and DNA-protein crosslinks by measuring the reduction in the elution in alkali of single stranded DNA from membrane filters by the linking of DNA strands or DNA and protein by the bifunctional reaction of cisplatinum with DNA and protein in the cell. This emphasis stemmed from early work showing superior interstrand crosslinking by cisplatinum over that by transplatinum (J.M. Pascoe et al, 1974) and also the correlation of interstrand crosslinking with cisplatinum toxicity (L.A. Zwelling et al, 1979a) although these crosslinks represent only approximately 1% of DNA adducts (J.J. Roberts et al, 1982).

Work by Zwelling and his colleagues mainly with L1210 cells has shown correlations between resistance (as well as toxicity) and DNA interstrand crosslinks by alkaline

elution (L.A. Zwelling et al, 1981; L.C. Erickson et al, 1981). In addition thiourea could inhibit toxicity and crosslinking (L.A. Zwelling et al, 1979b). However others have suggested that the crosslinking is simply related to dose of platinum (M.C. Strandberg et al, 1982). In L1210 sublines 20, 30 and 50-fold resistant to cisplatinum, the same degree of crosslinking was found at a particular drug dose for all three lines. Although the L1210 parent line showed equivalent crosslinking at a 5-fold lower dose, in agreement with the work of Zwelling, at equitoxic doses the resistant sublines had more crosslinks than the sensitive parent line. In both studies crosslinks increased to a maximum at 6 to 12 hours after drug exposure and then declined presumably due to DNA repair. Other workers have suggested that the persistence of these interstrand crosslinks for a considerable time could be important for differential toxicity in different cells (A.C.M. Plooy et al, 1984, 1985a).

The number of interstrand crosslinks appears to vary in proportion to the total DNA-adduct formation (A.C.M. Plooy et al, 1985b; R.J. Knox et al, 1986) and so their measurement appeared to be a good first step in assessing differential cisplatinum toxicity or resistance in the cell lines in this project. In addition, inhibition of DNA synthesis gives a broad indication of DNA damage. That inhibition and possible subsequent replication on a

damaged template may ultimately be the cytotoxic event (J.J. Roberts et al, 1980; R. Alazard et al, 1982). Thus these 2 assays were used here to initiate studies on resistance at the DNA level.

An entirely different line of experimentation was taken to explore possible gene amplification as a mechanism of resistance. Gene amplification has been implicated in resistance to a number of anticancer drugs although not so far to cisplatinum (R.T. Schimke, 1984). Together with Mr. Arthur Mitchell (of the MRC Clinical and Population Cytogenetics Unit) I decided to investigate this possibility using the technique of Roninson to detect new highly repeated DNA sequences in a drug resistant cell line (I.B. Roninson et al, 1984). For this purpose the most highly resistant line PE/01 CisPt^R was used since it might be expected to have the highest degree of amplification, if any had occurred.

6.1 Methods

Inhibitions of DNA synthesis by cisplatinum

Inhibition of DNA synthesis was measured by a pulse with [^{3}H]-thymidine at various times after cisplatinum treatment essentially as described previously for melanoma cells (P.G. Parsons et al, 1982). PE/01 and PE/04 cells were plated in 0.4mls medium at 8 x 10^4 and 5 x 10^4 cells/well respectively in 16mm wells of a 24 well plate

(Nunc, Gibco Ltd) and after 2 days another 0.3mls of medium added. This procedure produced even spread of cells across the well which were growing well by the third day when fresh medium was added and the cells treated with 5uM cisplatinum. At various times thereafter duplicate cultures were pulsed for 25 minutes with 15ul of 100uCi/ml [^{3}H-methyl]-thymidine (52 Ci/mmol, Amersham UK) before being harvested by trypsinisation and filtered onto GF/A glass fibre discs (Whatman) with water and 5% trichloroacetic acid. The filters were dried and counted in a liquid scintillation counter (Packard model 4430). Counts were expressed as percentage of control untreated wells pulsed and harvested at the same time.

DNA-interstrand crosslinks induced by cisplatinum

The alkaline elution technique developed by Kohn was used (K.W. Kohn et al, 1981). Some preliminary experiments used Polyvinyl Chloride filters (BSWP 02500, Millipore Corp.) with cell lysis by Sarkosyl (N-lauroyl sarcosine sodium salt, Sigma) but most experiments used polycarbonate membrane filters (2.0um pore size, Nuclepore Corp.) with lysis by SDS (sodium dodecyl sulphate, BDH Chemicals Ltd.) and using a proteinase K (Boehringer Mannheim) treatment step. The 25mm diameter filters were mounted in opaque Swinnex type filter holders (Millipore Corp).

Cells of PE/01, PE/04 or PE/01 CisPt^R cell lines were plated out in 25cm^2 tissue culture flasks (Nunc, Gibco Ltd) at 2 x 10^6 cells/flask in 4mls medium. Late the next day the cells were prelabelled with either 0.2uCi/ml [^{3}H-methyl]thymidine (52 Ci/mmol, Amersham) or 0.05uCi/ml [^{14}C-methyl]thymidine [54mCi/mmol, Amersham] in the medium overnight. The following day, after a change to fresh unlabelled medium for 1/2 hour, the cells were treated with cisplatinum for 2 hours and then incubated in medium without drug until being harvested at various later timepoints. Cells were harvested by trypsinisation and suspended in cold phosphate buffered saline (PBS). A cell pellet was spun down, resuspended in 1ml PBS and transferred to microfuge tubes (Eppendorf) which were kept cold on ice and shielded from light. Cells in these tubes were irradiated where necessary with 450 rads x-rays from a Cobalt-60 source at approximately 150 rads/minute. Control [^{3}H]-labelled cells not treated with cisplatinum were irradiated with x-rays and then added to cisplatinum treated [^{14}C]-labelled cells of the same cell line to act as an internal control in each elution.

Polycarbonate filters in Swinnex filter holders were washed with PBS, cells loaded on and washed through with more PBS under gravity or by suction from a multi-channel pump (Ismatec Instruments) at 0.4ml/minute (P. Bedford et al, 1984). Lysis buffer (2% SDS, 0.025M Na_2EDTA, pH 10.0)

was then added to drip through followed by lysis buffer containing 0.5mg/ml proteinase K. The outlet tubes were then clamped, more proteinase K lysis buffer added to fill the filter holders and protein degraded for approximately 50 minutes. This solution was then allowed to drip through before the alkaline elution buffer (approximately 0.1M tetrapropylammonium hydroxide, 0.02M EDTA (acid form), 0.1% SDS, pH 12.3) was added. Elution was carried out at a flow rate of approximately 0.03mls/minute, although there was some variation between pump channels, and 1 hour fractions collected in scintillation mini-vials in a Gilson model 202 fraction collector adapted to take 8 channels at a time. After the elution the filters were placed in scintillation vials, heated to 70° for 1 hour with 0.5mls 1N HCl, cooled and neutralized with 1.25mls 0.4N NaOH. The filter holders and line tubing were washed through with 0.4N NaOH and the washings collected and neutralized with 1N HCl. The fractions collected (1.5-2mls volume) were neutralized with 75ul 1N HCl. The filter, washings and fractions were counted on a liquid scintillation counter (Packard) after addition of Unisolve 1 (Koch-Light Ltd), a scintillant which can accommodate up to an equal volume of aqueous solution. All the samples were neutralized as above to improve counting efficiency. A sample of the lysis solution which had previously dripped through the filter was also counted to check for any loss of DNA before the elution but this never reached

more than 5% and usually less than 3% of the total counts.

The data is presented graphically as log % DNA retained on the filter versus time, using the elution of the [^{3}H]-labelled and irradiated control DNA as a modified time scale to compensate for any differences between filters and pump channels. I wrote a computer programme to assist in converting the data to this form. The apparent cross-link frequency expressed in rad-equivalents was calculated by the formula of Kohn.

$$P_c = \left(\sqrt{\frac{1 - R_0}{1 - R_1}} - 1\right) P_{BR}$$

where P_c is the cross-link frequency, P_{BR} the single strand break frequency from the radiation, R_0 the amount of DNA retained on the filter after irradiation and R_1 the DNA retained after drug treatment and irradiation. The cross-link frequency was calculated at 20% of the control irradiated DNA retained on the filter.

Repeated Sequence DNA Analysis

This work was done in collaboration with Mr. Arthur Mitchell of the MRC Clinical and Population Cytogenetics Unit who had an established interest in repeated DNA sequences in the human genome. Therefore this work done jointly with him would not have been possible without his knowledge and technical expertise and the use of restriction enzymes and DNA probes which he had available.

Knowledge on repeated sequence DNA has been recently reviewed (M.F. Singer, 1982).

DNA was prepared from confluent flasks of PE/01 (passage 81) and PE/01 CisPt^R (passage 111) cells by the method of Porteous (D.J. Porteous, 1985) with minor modifications. This follows the standard procedures of cell lysis, RNAase and pronase digestions, extractions with phenol and chloroform and spooling out DNA after ethanol precipitation. Two cycles of enzyme treatment, extraction and DNA precipitation were used and the DNA redissolved in 5mls of 10mM Tris buffer pH 7.5. The expected UV spectrum for purified DNA was observed and showed that the DNA obtained was at 760ug/ml for PE/01 DNA and 522ug/ml for PE/01 CisPt^R DNA. Normal human DNA, designated M5, was already available (A.R. Mitchell et al, 1985).

10ug samples of each of the three DNAs were digested with various restriction endonucleases in 30uls total volume for 6 hours and then run out on 1% agarose gels together with lambda HindIII markers (A.R. Mitchell et al, 1979) and transferred to nitrocellulose filters by the method of Southern (E.M. Southern, 1975). Restriction endonucleases used were HaeIII, BamHI, HindIII, EcoRI, HinfI, MboI, KpnI, MspI, HpaII, AluI and BglII. The DNAs on the nitrocellulose filters were hybridized using the p82H probe for repeated sequences in centromeric regions of

human chromosomes (A.R. Mitchell et al, 1985) and also the probe (D.J. Porteous, 1985) for the dispersed human repeated sequence KpnI (J.W. Adams et al, 1980; B. Shafit-Zagardo et al, 1982) and the filters exposed to X-ray film to visualise bands of hybridized DNA.

The technique of Roninson to detect repeated and amplified DNA fragments, where the genome being tested in effect provides the probe for any sufficiently repeated sequence, was also tried (I.B. Roninson, 1983; I.B. Roninson et al, 1984). In a first experiment 60ug of each of the 3 DNAs was digested with BamHI (60 units of enzyme in 180uls final volume for 6 hours). 1.5ug DNA from these digests was labelled as tracer DNA using T4 DNA polymerase for 30 minutes in the exonuclease reaction before addition to 200uCi [α-^{32}P]dCTP and the other deoxynucleotide triphosphates for resynthesis. The tracer DNA of each sort (M5, PE/01, PE/01 CisPt^{R}) was then co-electrophoresed with unlabelled "driver" DNA (15ug) of each sort from the above digests. The gel was subjected to 2 cycles of in-gel renaturation and S_1 nuclease digestion with hybridization for 15 hours and then 2 hours in the 2 cycles respectively and was then dried down and autoradiographed. A second experiment was done in similar fashion except the T4 DNA polymerase exonuclease reaction was continued for only 6 minutes to try to reduce the background on the gel.

6.2 Results

Inhibition of DNA synthesis by cisplatinum

PE/01 and PE/04 cells in log culture were treated with 5uM cisplatinum and pulsed for 25 minutes with [^{3}H]-thymidine at various times thereafter as a measure of DNA synthesis. Cisplatinum exposure was continued for the duration of the experiment. The results are shown in Figure 46. Inhibition gradually increased with time. PE/01 showed a small but consistently increased inhibition of DNA synthesis compared with PE/04 over all of the 24 hour time course.

DNA-interstrand crosslinks induced by cisplatinum

These experiments used the alkaline elution technique developed by Kohn and his colleagues (K.W. Kohn et al, 1981). An initial experiment demonstrated equal elution of DNA from PE/01 and PE/04 cells after irradiation with 500 rads of x-rays as shown in Figure 47. Since variations in the elution rate between different channels of the multichannel pump were apparent, the volume of the eluant was used as an adjusted time scale to compare the elution rate from different filters. In later experiments with cisplatinum treatment, an internal control of irradiated but otherwise untreated cells labelled with [^{3}H]-thymidine was used in each elution and its elution rate served as an adjusted time scale to compare DNA elution from different filters.

Figure 46 Cisplatinum Inhibition of DNA Synthesis

PE/01 ×

PE/04 ●

Cells were treated with 5uM cisplatinum and DNA synthesis estimated by ^{3}H -thymidine incorporation at various times thereafter.

Cisplatinum was left on for the duration of the experiment.

Figure 46

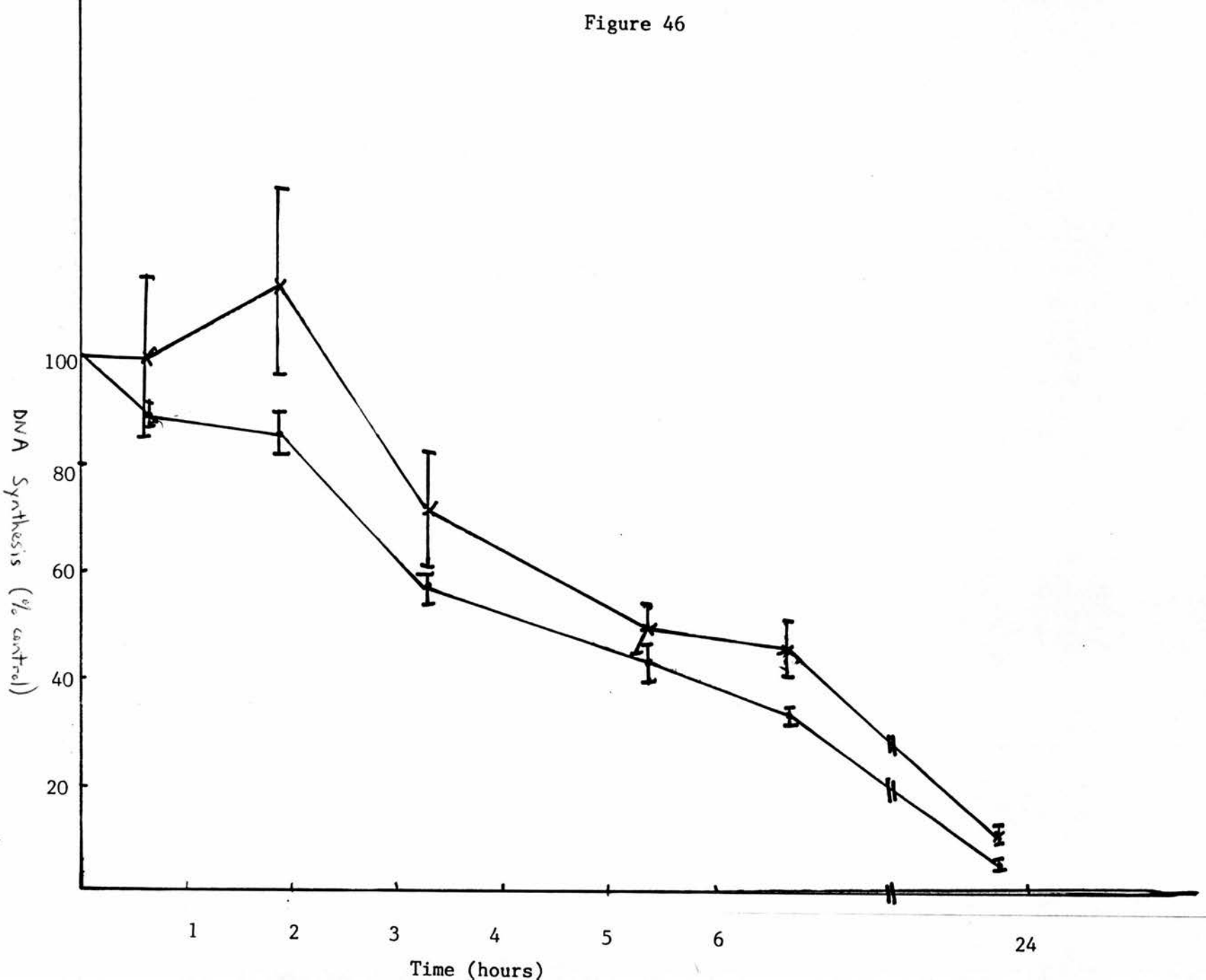
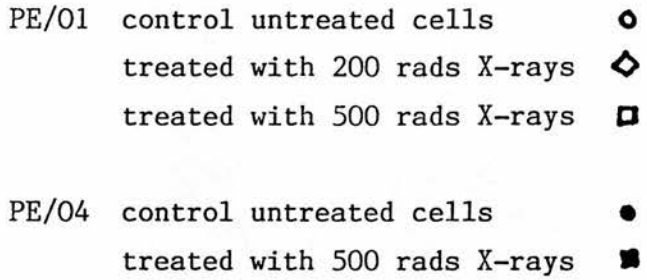
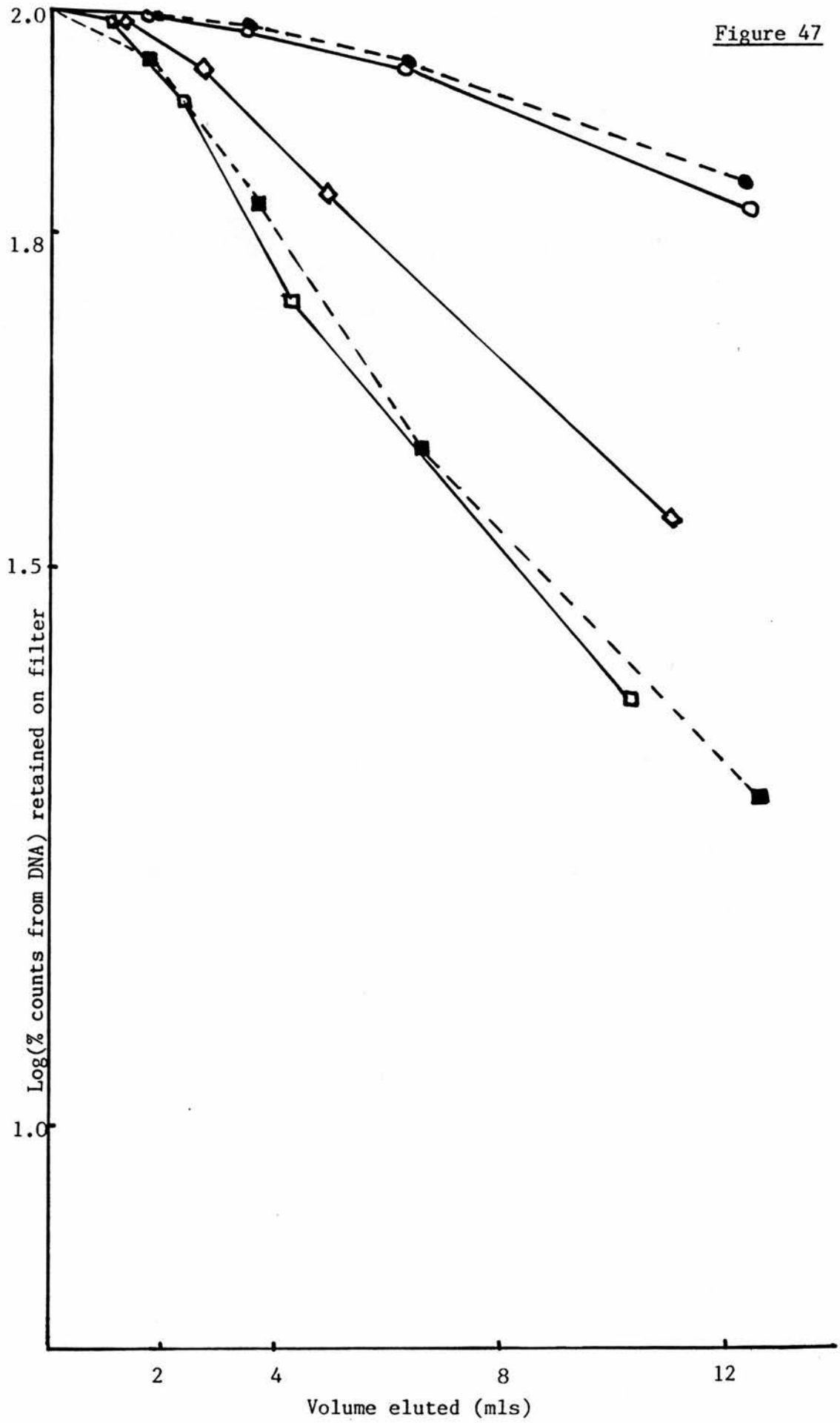


Figure 47 Alkaline elution of X-irradiated cells



Cells were harvested into cold PBS, irradiated from a Cobalt 60 source, and eluted through polyvinyl chloride filters with tetramethyl ammonium hydroxide + 0.02M EDTA pH 12.3 after cell lysis on the filter with 0.2% sarkosyl, 2M NaCl, 0.02M Na_2EDTA pH 10.0.

Figure 47


2.0
1.8
1.5
1.0
Log(% counts from DNA) retained on filter
2
4
8
12
Volume eluted (mls)

Preliminary experiments showed a decrease in the elution rate of DNA from PE/01 and PE/04 cells treated with 2uM cisplatinum for 6 hours indicative of DNA-protein cross-links and/or DNA interstrand crosslinks. Therefore in time course experiments a concentration of 5uM cisplatinum was chosen with drug exposure being for 2 hours with subsequent incubation without drug. Interstrand cross-linking expressed in rad-equivalents, over a 24 hour period is shown in Figure 48 for the cell lines PE/01 and PE/04 with results from 3 experiments shown. Approx-imately 10% of DNA from unirradiated cells with or without drug exposure eluted from the filter at the retention time used to calculate crosslinking frequency. No evidence for DNA single strand breaks caused by cisplatinum was seen. Since only 8 filters could be eluted at one time it wasn't possible to do an extensive timecourse with both cell lines in the one experiment and the variation between experiments was larger than expected. In only one of the three experiments were PE/01 and PE/04 treated together with cisplatinum and eluted on the same day. In light of this variation, little difference between the two cell lines at 5uM cisplatinum concentration was observed, either in the development, extent or repair of interstrand crosslinks. Maximum crosslinking appeared to occur at 6 to 12 hours as reported by others (L.A. Zwelling et al, 1979a; A.C.M. Plooy et al, 1984) but little repair of these crosslinks was evident.

Figure 48 Time course of Development of DNA Interstrand Crosslinks induced by Cisplatinum

Experiment 1. PE/01 ○

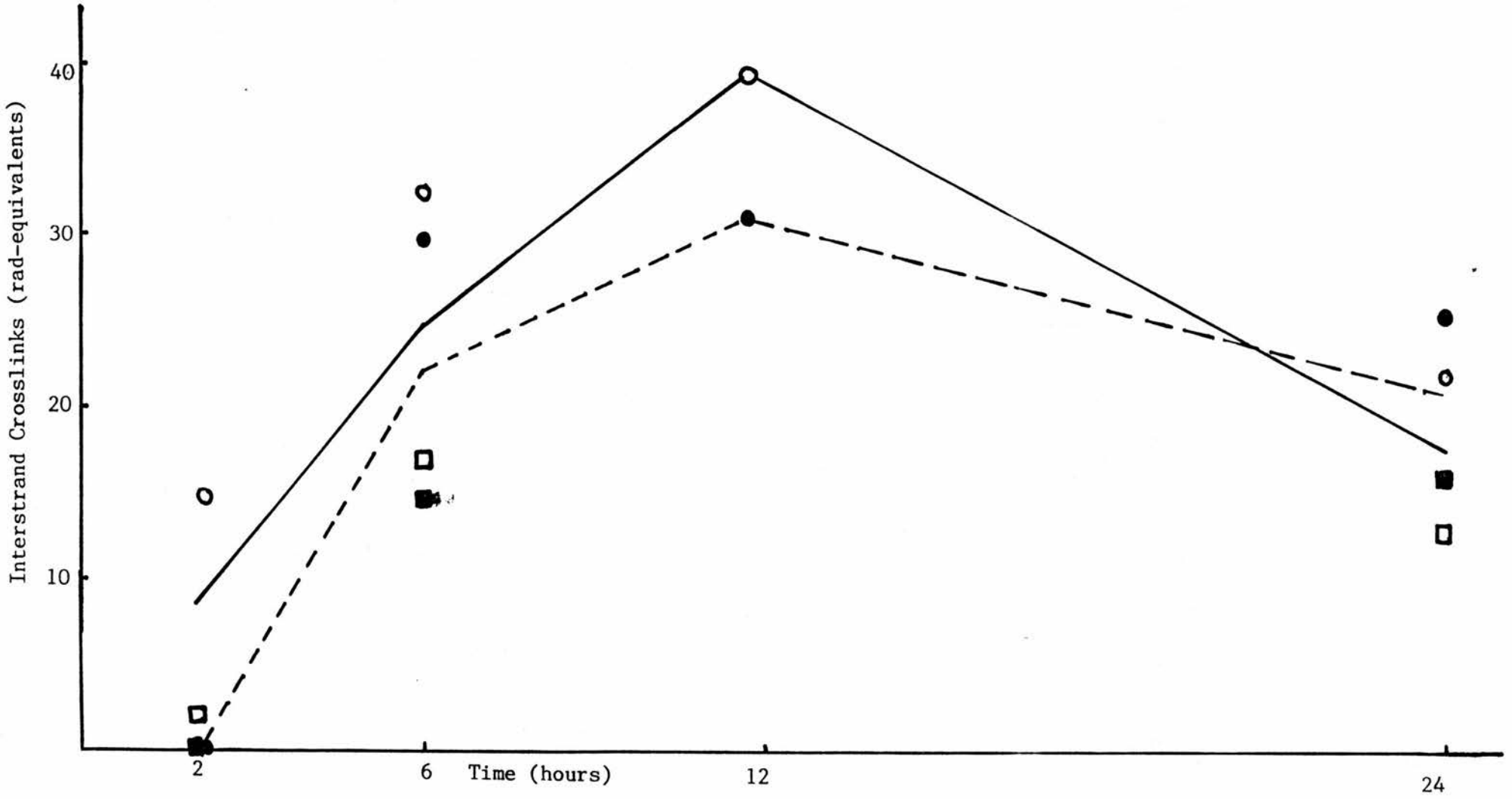
Experiment 2. PE/04 ●

Experiment 3. PE/01 □, PE/04 ■

(line drawn between mean values PE/01 ——
PE/04 - - - -)

Cells were treated with 5 uM cisplatinum for the first 2 hours of the time course and then harvested at various times later for assessment of DNA interstrand crosslinking by alkaline elution.
Crosslinking is expressed in rad-equivalents by the method of Kohn (see Methods section).

Figure 48


Interstrand Crosslinks (rad-equivalents)
40
30
20
10
2
6
Time (hours)
12
24

It was then decided to check the dose response of each cell line in forming interstrand crosslinks to increasing doses of cisplatinum. Cells were exposed to cisplatinum for 2 hours and incubated for a further 4 hours without drug (i.e. 6 hours total incubation as above) before being harvested for the alkaline elution assay. The elution curves for 3 different experiments are reported in Figures 49, 50 and 51 with each figure drawn on the same scale. Again some variation between experiments can be observed. However, a clear dose response of crosslinking with increasing dose of cisplatinum above 5uM is apparent in each experiment and at the higher doses of 20uM and 50uM cisplatinum PE/01 showed greater interstrand crosslinking than PE/04. In addition in the experiment shown in Figure 51 the interstrand crosslinking at these doses in the resistant subline PE/01 $CisPt^R$ is considerably less than either PE/01 or PE/04. Apparent crosslink frequencies calculated in rad-equivalents from Figures 48-51 are shcwn in Table 14. With the low doses of cisplatinum (1uM and 5uM) it was more difficult to take accurate data from the graphs. Mean values were 12, 67 and 158 rad-equivalents for PE/01 and 19, 38 and 98 rad-equivalents for PE/04 at doses of 5, 20 and 50uM cisplatinum respectively. These values are of the same order of magnitude as reported by others (L.A. Zwelling et al, 1981; L.C. Erickson et al, 1981). As also previously reported a linear increase in crosslinking with cisplatinum dose was also apparent. The

Figure 49 Alkaline elution - PE/01 and PE/04
1, 5, 20 uM cisplatinum

1 uM cisplatinum	PE/01	○	PE/04	●
5 um cisplatinum	PE/01	□	PE/04	■
20 um cisplatinum	PE/01	◇	PE/04	◆
control unirradiated	PE/01	×	PE/04	+

All cells irradiated with 450 rads X-rays (except control cells) and treated with proteinase K after cell lysis on the filter.
Elution is plotted against the elution of ^{3}H-labelled control cells of the same cell line irradiated with 450 rads X-rays and added with the ^{14}C-labelled cisplatinum treated cells on each filter. The irradiation introduces DNA single strand breaks and DNA interstrand crosslinks are evidenced by a reduction in elution rate of the irradiated DNA.

Figure 49

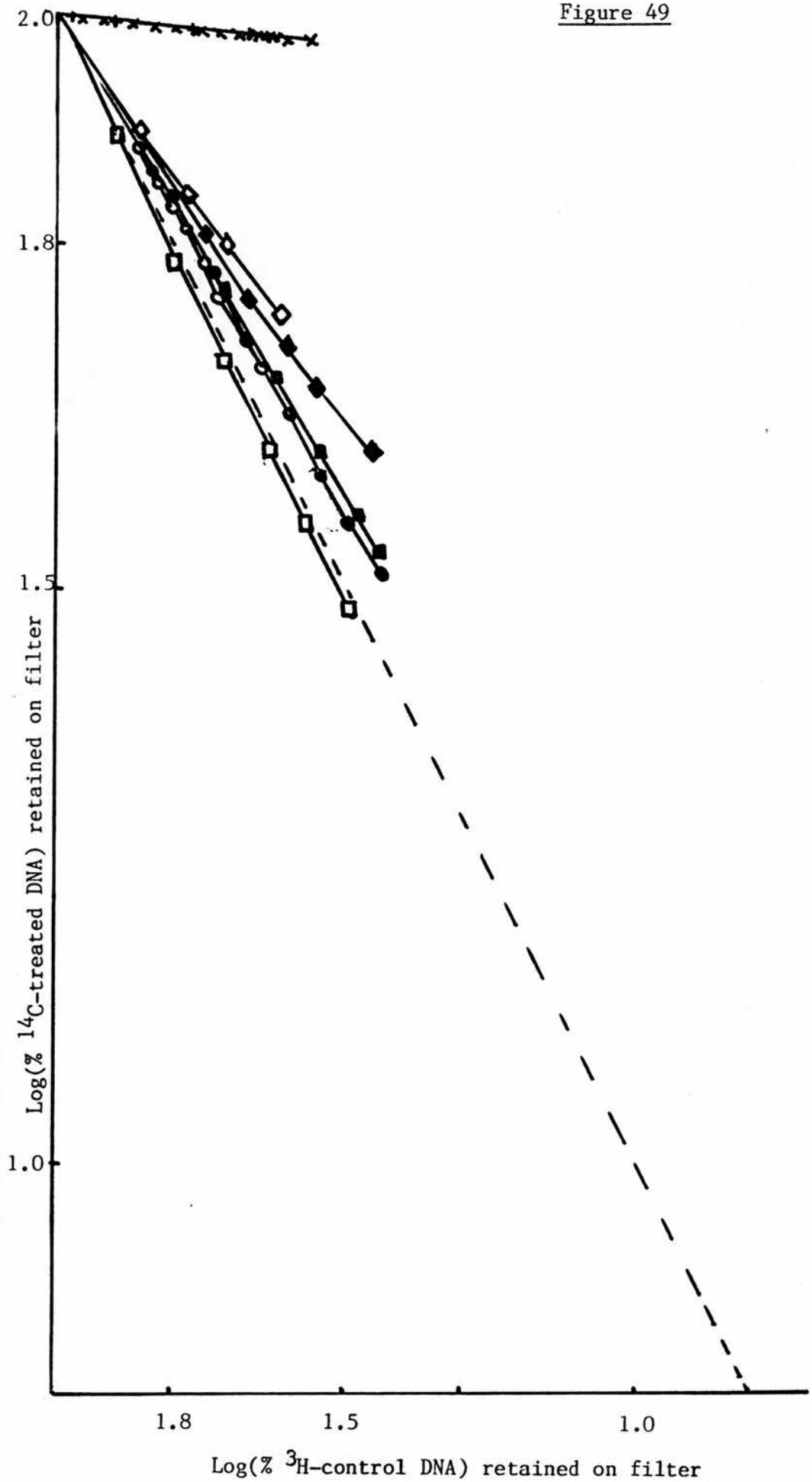

2.0
1.8
1.5
1.0
Log(% 14C-treated DNA) retained on filter
1.8
1.5
1.0
Log(% 3H-control DNA) retained on filter

Figure 50 Alkaline elution - PE/01 and PE/04
5, 20, 50 uM cisplatinum

5 uM cisplatinum	PE/01	□	PE/04	■
20 uM cisplatinum	PE/01	◇	PE/04	◆
50 uM cisplatinum	PE/01	○	PE/04	●
50 uM cisplatinum but unirradiated	PE/01	×		
Control unirradiated	PE/04	+		

Elution after 450 rads X-rays and proteinase K treatment, as detailed in Methods section and legend to figure 49.

Figure 50

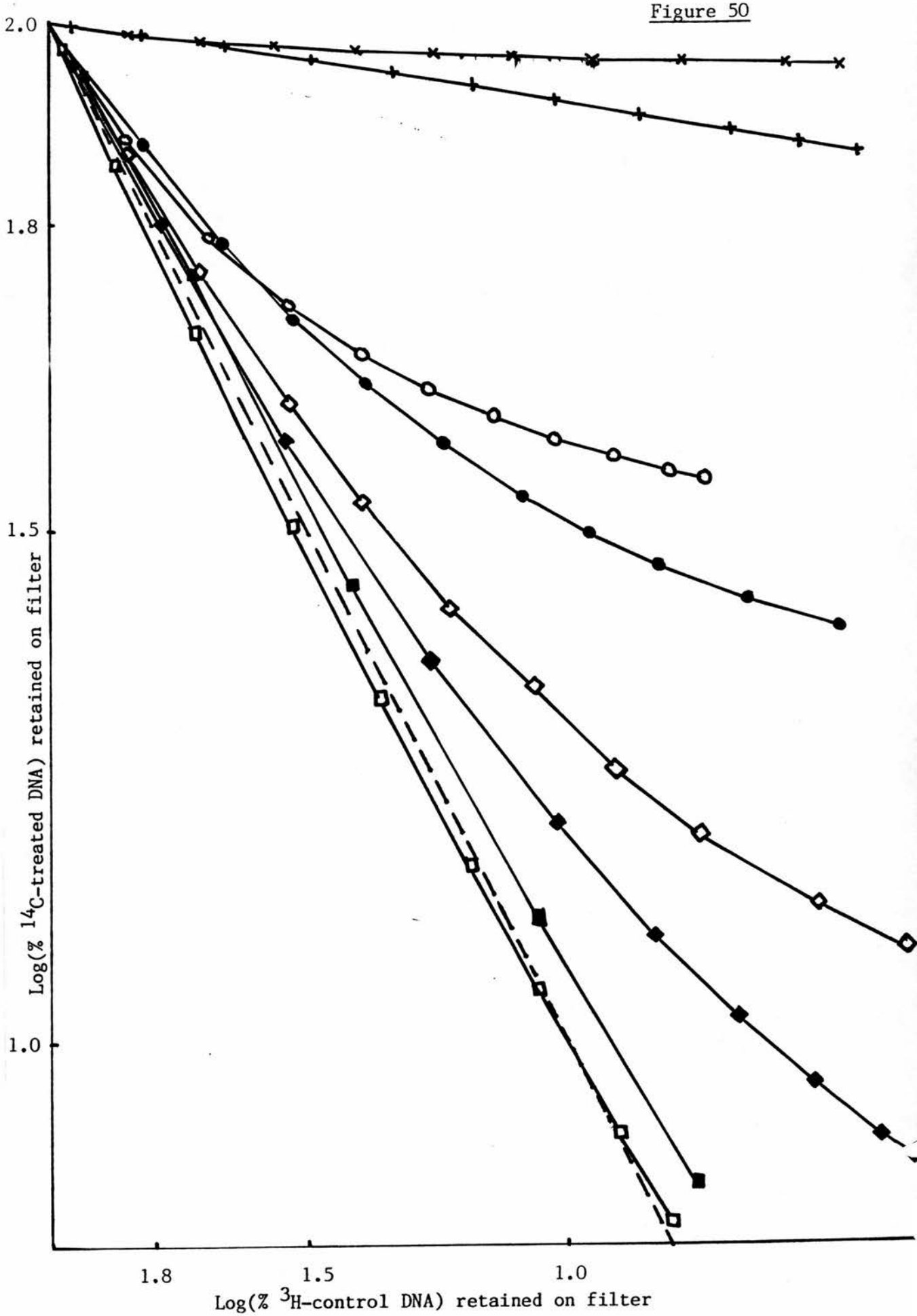
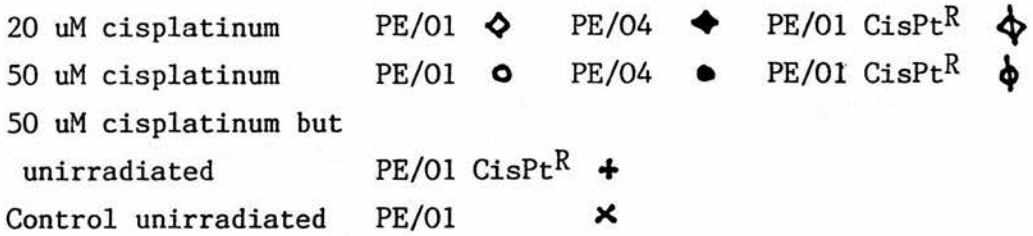

2.0
1.8
1.5
1.0
Log(% ^{14}C-treated DNA) retained on filter
1.8
1.5
1.0
Log(% ^{3}H-control DNA) retained on filter

Figure 51 Alkaline elution - PE/01, PE/04 and PE/01 CisPt^R 20, 50 uM cisplatinum



Elution after 450 rads X-rays and proteinase K treatment, as detailed in Methods section and legend to figure 49.

Figure 51

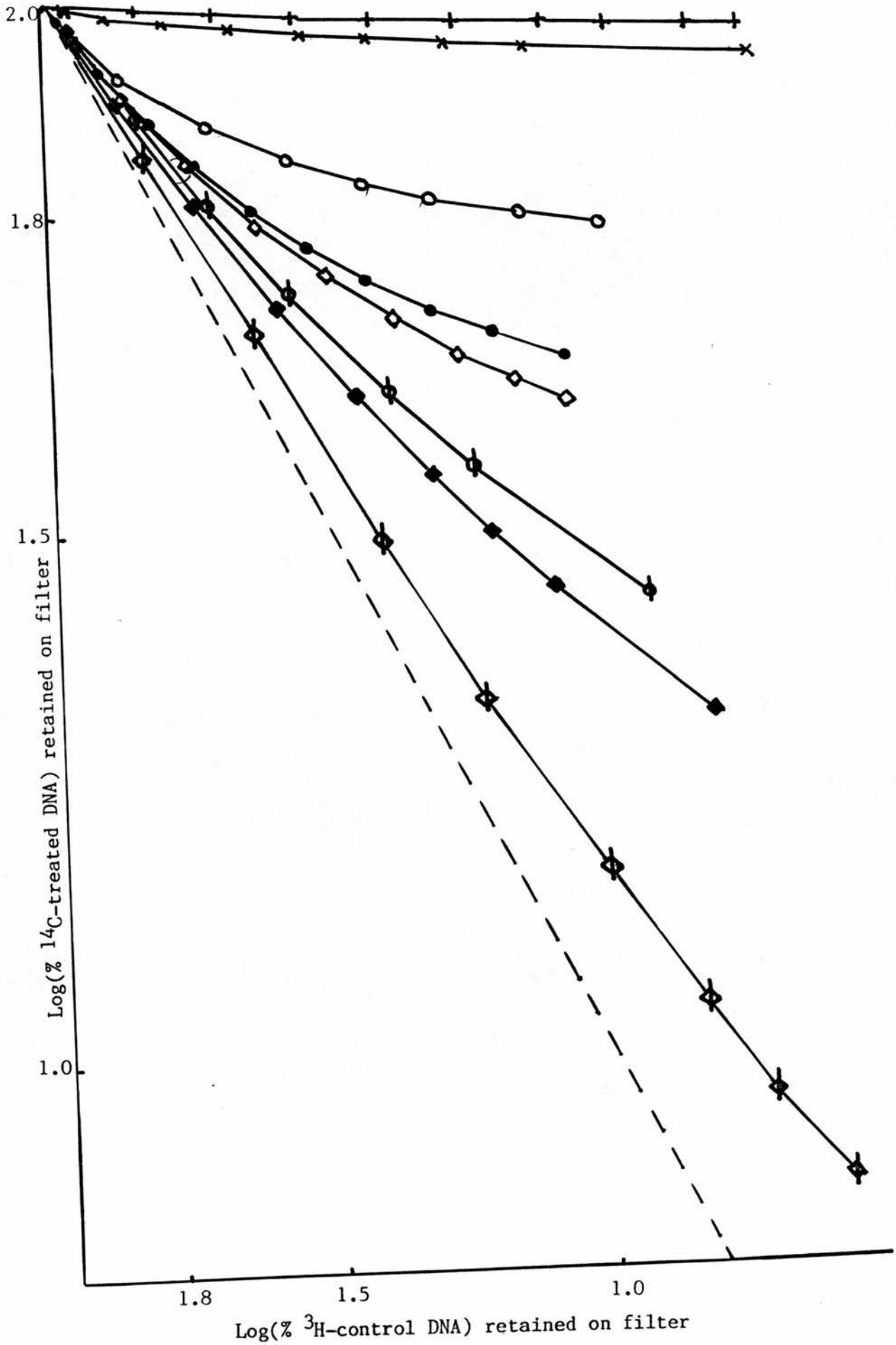

2.0
1.8
1.5
1.0
Log(% 14C-treated DNA) retained on filter
1.8
1.5
1.0
Log(% 3H-control DNA) retained on filter

TABLE 14

DNA Interstrand-Crosslinking

		Crosslinking (rad-equivalents)[a]			
		1uM	5uM	20uM	50uM Cisplatinum
PE/01	(48)*	-	32,17[c]	-	-
	(49)[d]	21	0	65	-
	(50)	-	0	30	91
	(51)	-	-	105	225
PE/04	(48)	-	30,15[c]	-	-
	(49)[d]	25	26	49	-
	(50)	-	6	15	73
	(51)	-	-	49	122
PE/01 CisPt^R	(51)	-	-	15	64

* Numbers refer to the figure in the text which shows the experiment from which the crosslink frequency has been calculated.

(a) Crosslinking frequency 4 hours after a 2 hour exposure to cisplatinum.

(c) Second reading shown here calculated from the same experiment for PE/01 and PE/04 of the 3 experiments shown in figure 40.

(d) Calculated at 48% instead of 20% of [^{3}H] DNA retained on the filter since fractions eluted more slowly in this experiment.

slope of a linear regression line of crosslinking versus drug dose was some 1.9x higher in PE/01 than PE/04, indicative of more interstrand crosslinks being formed per increment of cisplatinum dose increase in that cell line.

Repeated Sequence DNA Analysis

Two probes for specific repeated sequence DNA were available, one for sequences shown to be located in centromeric regions of all human chromosones (p82H, A.R. Mitchell et al, 1985) and one for the dispersed Kpn repeated sequence (D.J. Porteous, 1985; B. Shafit-Zagardo et al, 1982). Both were used since it is possible that new or rearranged amplified sequences could be found with other repeated sequence DNA (as shown for methotrexate resistance with amplified sequences associated with satellite DNA (C.J. Bostock et al, 1980)) and thus shift the position of DNA restriction fragments seen on agarose gels. The DNA from the PE/01 and PE/01 CisPt^R cell lines was compared together with normal human DNA (A.R. Mitchell et al, 1985). DNA digested with HaeIII, BamHI, HindIII, EcoRI, HinfI, MboI, KpnI, MspI, HpaII, or AluI restriction endonucleases was analysed with the p82H probe. A number of differences were observed between the M5 normal human DNA and the DNA from the 2 tumour cell lines. New DNA bands and missing or very faint bands were seen in the tumour lines with most of the restriction endonucleases used except KpnI which simply gave a smear of DNA and MspI

where most of the DNA hybridized on the gel was in the high molecular weight region. On the other hand little DNA hybridized in the high molecular weight region of the gel with HinfI with the normal DNA but there was a considerable smear of DNA for PE/01 and PE/01 CisPt^R. Some possible differences between PE/01 and PE/01 CisPt^R appeared to be simply due to variation in intensity of bands possible due to loading differences and were not seen in a repeat experiment. Thus no new p82H hybridizing bands were observed in PE/01 CisPt^R compared with PE/01. With the Kpn probe enzyme digests with KpnI, HindIII and BglII only were examined. Again some marked differences between the M5 normal human DNA and the DNA of the 2 cell lines were observed but no differences seen between the 2 cell lines.

The technique developed by Roninson (I.B. Roninson et al, 1984) was thought more likely to show any differences between the cell lines. It relies on in situ denaturation and renaturation of DNA restriction fragments separated on an agarose gel and displays amplified sequences as novel bands which are missing from the control DNA. In this way it can confirm the presence of DNA sequence amplification without any prior knowledge of the nature of the amplified sequence itself. However, a first experiment using this technique gave a high background smear of DNA down the gel which could not be removed. This made detection of bands

of repeated sequence DNA difficult and was probably due to labelling of the short interspersed Alu repetitive sequences present in human DNA (C.M. Houck et al, 1979). As discussed by Roninson (I.B. Roninson, 1983) labelling may need to be limited to the terminal portion of the restriction fragment to avoid this problem and consequently the T4 DNA polymerase exonuclease reaction was reduced from 30 minutes to 6 minutes in a second experiment. In addition in the first experiment radioactivity in the M5 normal human DNA tracer was 10-fold less than in the other DNA tracers and hence these lanes on the gel could not be visualised effectively. A diffuse band at low molecular weight appeared to show some difference between PE/01 CisPt^R and PE/01 in some lanes but not in others. In the second experiment the band to background ratio was much improved. However, here the PE/01 CisPt^R tracer had much reduced radioactivity and these lanes could not be visualised so no comparison of the 2 cell lines was possible. No significant differences were observed between the M5 and PE/01 DNA although there was some difference in the density of bands at low molecular weight.

6.3 Discussion

In this study the measurement of DNA interstrand crosslinking by cisplatinum correlated with resistance to drug toxicity in the 3 cell lines PE/01, PE/04 and PE/01

CisPt^R. Reduced crosslinking at a particular dose of cisplatinum correlated with increased resistance, in agreement with the data of Zwelling (L.A. Zwelling et al, 1981) and more recent data in a preliminary report with unrelated ovarian cancer cell lines (B.G. Ward et al, 1985). However, the difference in the amount of crosslinking between the cell lines, particularly for PE/01 CisPt^R may not be enough to completely explain the degree of resistance observed. Other factors would appear to be important as well, such as mono-adduct quenching, other lesions, or effects before the drug reaches the nucleus, as suggested by others in similar studies based on the alkaline elution technique (M.C. Strandberg et al, 1982; K. Micetich et al, 1983) although crosslinking may not necessarily need to show a linear correlation with the extent of resistance to explain that resistance. Others have emphasised that the extent of total platinum bound to DNA is correlated with drug toxicity (J.J. Roberts et al, 1980; R.J. Knox et al, 1986) and interstrand crosslinks may simply correlate with Pt-DNA binding (as in the study of A.C.M. Plooy et al, 1985b) or some other toxic DNA lesion.

Some variation in the estimate of crosslinks at a range of elution end points was observed, at variance with the reports of Kohn´s group (R.A.G. Ewig et al, 1978) but seen by others (M.C. Strandberg et al, 1982), although the

relation between different crosslink estimates within an experiment remained very similar at different retention times. The crosslink frequency was also calculated according to the method of Plooy which mathematically should give a better estimate since crosslinks are assumed to slow rather than stop elution of DNA from the filter at the retention time chosen (A.C.M. Plooy et al, 1984). This method calculates DNA retention at 50% control irradiated DNA retained on the filter. Since both methods need to estimate the retention of treated DNA graphically, it can be seen in my experiments that differences between different cell lines or treatments appear small and thus difficult to estimate at 50% control DNA retained compared with 20% control DNA retained. Also since the lines graphed are slightly curved rather than straight, almost no difference at 50% control DNA retained can change to a clear difference when more DNA has eluted (as also discussed by M.C. Strandberg et al, 1982). For these reasons the method of Kohn was preferred, since the two methods also gave qualitatively similar results although as reported by Plooy his method gives crosslink frequencies approximately 2-fold higher than the Kohn method. Thus the frequencies obtained can only be regarded as relative values and so no attempt was made to compute absolute values of crosslinks/10^9 daltons DNA or cross-links/cell as quoted by some authors (M.F. Pera et al, 1981; A.C.M. Plooy et al, 1984). Only the estimated

crosslink frequencies according to the Kohn method have been reported here. At low cisplatinum doses interstrand crosslinking was difficult to measure (figures 49 and 50 have one curve below the irradiation only curve which mathematically would lead to minus crosslinking values as also shown by L.C. Erickson et al, 1981). However the crosslinking frequencies shown in Table 14 are of the same order of magnitude as reported by others (L.A. Zwelling et al, 1981; L.C. Erickson et al, 1981) and show a linear increase with cisplatinum dose as also previously reported.

Although interstrand crosslinks are a minor DNA lesion caused by cisplatinum (J.J. Roberts et al, 1982; A. Eastman, 1985) they may nevertheless have a biological role if DNA replication cannot proceed past a lesion involving both DNA strands particularly if the lesion is difficult to repair and thus persists in the DNA (A.C.M. Plooy et al, 1985a). In addition inhibition of interstrand crosslink formation by thiourea (L.A. Zwelling et al, 1979b) or diethyldithiocarbamate (D.L. Bodenner et al, 1986) was correlated with reduced cytotoxicity of cisplatinum and when these thio compounds were added after crosslinks had formed no reduction in crosslinks or toxicity was observed. The kinetics of the formation and repair of interstrand crosslinks has been extensively studied (K. Micetich et al, 1983; A.C.M. Plooy et al,

1985a). Murray has investigated their persistence in vivo and shown variations amongst normal mouse tissues and in 2 different tumours (D. Murray et al, 1985). A recent preliminary report showed a correlation of interstrand crosslinks with drug sensitivity in 2 bladder carcinoma cell lines but less crosslinks in a more sensitive testicular tumour cell line at an equitoxic dose indicating differences between tissues which may be important (P. Bedford et al, 1986). The mechanism of the slow formation of interstrand crosslinks is uncertain but it can occur even in DNA preparations in buffer (A. Eastman, 1985; R.J. Knox et al, 1986) and is presumably due to the appropriate conformational changes in the secondary structure of the DNA after a mono-adduct is formed (A. Eastman, 1985). Since DNA-protein crosslinks and intrastrand crosslinks are formed rapidly (K. Micetich et al, 1983; A.C.M. Plooy et al, 1985) other mono-adducts must be either repaired or be in positions where no other reactions are possible to form stable products in the meantime. It is possible that these mono-adducts are stabilised temporarily by other ligands such as phosphates (E. Segal et al, 1985) or the exocyclic ring oxygen at the 6 position of guanine (S.J. Lippard, 1982). A recent preliminary report has suggested that a late step in the repair of a rare DNA lesion, such as an interstrand crosslink, may be important in sensitive versus resistant Walker 256 carcinoma cells (J.J. Roberts et al, 1986).

Interstrand crosslinking is known to be important for other agents like the bifunctional alkylating agent melphalan (P.G. Parsons, 1984). Quantitatively minor lesions have also been shown to be important for other agents, particularly in the well characterised case of monofunctional alkylating agents and the O^6-alkylguanine lesion (D.B. Yarosh, 1985).

Since cis-bifunctionality is important to cisplatinum toxicity, intrastrand crosslinks which can be formed by cis- but not trans- platinum have received increasing recent attention (A.C.M. Plooy et al, 1985; E. Reed et al, 1986). Reed and his colleagues have suggested a positive correlation between formation of DNA intrastrand cross-links in DNA of buffy coat cells from peripheral blood of testicular and ovarian cancer patients and response to therapy. Although the intrastrand crosslink is the major DNA lesion caused by cisplatinum (A. Eastman, 1985; A.C.M. Plooy et al, 1985) its true biological significance is yet to be assessed. Filipski has suggested it is the important toxic lesion for λphage inactivation by cisplatinum (J. Filipski et al, 1980). Chemically 2 neighbouring guanines in a DNA strand is the preferred site for cisplatinum binding (A. Eastman, 1983). It may inhibit DNA replication (A.L. Pinto et al, 1985a) or it may be a difficult lesion to repair (A.C.M. Plooy et al, 1985; R.B. Cicarell et al, 1985) and its role awaits further elucidation.

The importance of the various DNA repair pathways in repairing cisplatinum-DNA damage is unclear, partly due to the complexity and the lack of knowledge in detail in mammalian cells (M. Fox, 1984). Cisplatinum mono-adducts are repaired most efficiently (A.C.M. Plooy et al, 1985) and their repair or "quenching" before more toxic (or difficult to repair) lesions are formed may be important in cellular resistance to cisplatinum (K. Micetich et al, 1983). Excision repair and postreplication repair pathways have both been implicated (J.J. Roberts et al, 1979). Some authors have emphasised the role of excision repair (I. Husain et al, 1985) and its possible role in removing intrastrand crosslinks (D.J. Beck et al, 1985). Others have recognised differences in responses to UV or cisplatinum damage indicative of differences in their "excision" repair (H.N.A. Fravel et al, 1978; M. Germanier et al, 1984). More than one pathway may be involved. Excision and recombinational repair together were emphasised by Hannan, possibly necessary to repair interstrand crosslinks (M.A. Hannan et al, 1984). Postreplication repair has been implicated through the potentiation of cisplatinum toxicity by caffeine (J.J. Roberts et al, 1979, 1986a). Based on this work Roberts has suggested that the important final genotoxic lesion is a double strand break formed upon attempted DNA repair or replication on a damaged template (J.J. Roberts et al, 1986a). Recovery from the cytotoxic effects of

cisplatinum has been observed in normal human fibroblasts after confluent holding (E.H.A. Poll et al, 1985) also suggesting the importance of repair before DNA replication. The DNA damage probably causes the inhibition of DNA synthesis observed after cisplatinum treatment. In this project a small increase in inhibition of DNA synthesis was observed in the sensitive cell line which supports the evidence of increased DNA damage seen in the alkaline elution assay. A similar correlation of low resistance (2-fold) and increased interstrand crosslinking and inhibition of DNA synthesis (2-fold for both) has been observed in 2 unrelated bladder transitional cell carcinomas (P. Bedford et al, 1986a).

Further experiments with the alkaline elution assay could define the kinetics and repair of interstrand crosslinks. DNA-protein crosslinks could be investigated particularly at short times as an indicator of the amount of active platinum species reaching the nucleus (as suggested by J.M. Ducore, 1986). The importance of these lesions needs to be investigated in other cell lines and with the other platinum analogues cross-resistant and not cross-resistant to cisplatinum. The recognition of intrastrand crosslinks would also be important but this would rely on obtaining the appropriate antibodies (A.M.J. Fichtinger-Schepman et al, 1985a). Total platinum binding to DNA can be assessed using atomic absorption spectroscopy as in Chapter 4 and

as described for ovarian cancer cells by Knox (R.J. Knox et al, 1986).

In the repeated sequence DNA analysis no evidence for gene amplification was obtained. It is not surprising that some differences between the normal human DNA and the 2 cell lines were obtained since it involved DNA from different individuals and the 2 cell lines represent tumour DNA. The technique of Roninson needs gene amplification to approximately 15-30 copies before it would be detected (I.B. Roninson et al, 1984) and it is possible that the PE/01 CisPt^{R} sub-line which is 25-fold resistant might not have enough amplified copies if gene amplification had in fact occurred. Others have also since used this technique (A.T. Fojo et al, 1985; P. Meltzer et al, 1986) and its particular advantage is in detecting amplification when the nature of the amplified sequence in question is unknown so it may therefore be a very useful technique for further work. As reviewed by Stark (G.R. Stark, 1986) there is no data as yet to implicate gene amplification (so far identified with methotrexate and the pleiotropic drug resistant phenotypes) as a mechanism of resistance to cisplatinum but this possibility will need to await further investigation.

7. General Discussion

7.1 Development of an ovarian tumour model for drug resistance studies

This project has used 2 human ovarian carcinoma cell lines derived from ascites at different times from the one patient as a basis for a model of drug resistance in ovarian cancer. As the project progressed the model has been characterised in detail and extended with resistant sublines derived in vitro and other cell lines derived from further tumour, particularly ascites, samples which were collected from patients referred to the Department of Clinical Oncology. Cross-resistance to a variety of drugs has been investigated and emphasis has been placed on mechanisms of resistance to cis-platinum which has become the leading drug in the chemotherapy of ovarian cancer. The specific results have been discussed in the light of the current literature at the end of the relevant chapters.

The philosophy of the project has been to build up a model which was as close to the clinical situation as possible. Therefore only human ovarian cancer cells have been used and further ascites samples continue to be collected and frozen in liquid nitrogen when not used immediately. This bank of ascites cells will be useful for drug sensitivity testing or biochemical tests suggested by the work on the derived cell lines. I have also attempted to avoid

possible variability in results due to continued passaging of cell lines (discussed in Chapter 3) and again different passages, from the earliest passages where there were enough cells, have been frozen in liquid nitrogen for future reference as needed. While I was keen to take the work back from the cell lines to the original ascites the project did not get this far in the time available. Large numbers of cells and repeat experiments are often necessary in investigating mechanisms of resistance and this limited this work to the cell lines. Since the stored ascites cells represent a precious and, for any one patient with particular characteristics of tumour, treatment and response, limited resource they have been kept until now for further work envisaged in this laboratory. One area for future study would be the possibility of using the alkaline elution assay (with a fluorescent dye to measure the DNA, P. Bedford et al, 1984) to measure cisplatinum induced DNA crosslinking in ascites samples either in vitro or after patient treatment in vivo.

Obviously the postulate of resistance to cisplatinum acquired in the patient from which the PE/01, PE/04 and PE/06 cell lines were derived would be greatly strengthened if differences in drug sensitivity in the ascites samples (from which the cell lines were derived) could be shown. The work of Trent and his colleagues has

demonstrated cytogenetic variation between ovarian ascites samples and derived cell lines (J.M. Trent et al, 1985). Two ascites 9 months apart from 1 patient showed a very similar karyotype. A cell line derived from the first ascites showed a different karyotype (at passage 3 and which remained stable in culture) and a cell line derived from the second ascites represented a minor population in the ascites (and its karyotype changed in culture). However most of the karyotypic alterations of the tumour were conserved in the cell lines. (R.N. Buick et al, 1985). Rather more karyotypic changes between primary tumours and cell lines have been observed for human gliomas and these occurred in the first weeks of culture (J.R. Shapiro et al, 1985). Variaticns in chemosensitivity between different clones were also observed. Near diploid clones appeared to be more stable karyotypically but could be rapidly overgrown by faster growing hyperdiploid cells which were also more sensitive to BCNU treatment. Greater variation in karyotype was observed in explant cultures than in immediate clonal cultures (J.R. Shapiro et al, 1981). Clearly culture of human tumour cells in vitro places them under a different selective environment which may change features representative of the tumour. In any case the PE/01, PE/04 and PE/06 cell lines represent a useful in vitrc model for investigating mechanisms of lcw level resistance. The similar resistance of PE/04 and PE/06

over PE/01 supports the idea of a change or selection induced by the patient's chemotherapy.

While collection of ascites continued I was particularly interested to obtain ascites from patients previously untreated with chemotherapy or radiotherapy and to follow their progress where this was possible. Cells from the first ascites or tumour sample provide a reference point of the tumour population before selection or alteration by drug treatment. Serial sets of samples from the same patient patient would help to analyse drug resistance acquired in vivo as distinct from the many studies of drug resistance where cell lines have been treated in vitro to give resistant sub-lines (T.C. Hamilton et al, 1984a). Four interesting multiple sets of ascites have been collected here (Chapter 2), of which time only permitted the PE/01 family of cell lines to be studied in detail as yet. One study by Buick and his colleagues has reported a series of 7 ascites from 1 patient (W.J. Mackillop et al, 1983) with cell lines reported from the first and last ascites (R.N. Buick et al, 1985) but drug sensitivity data has been reported only from the second cell line and not from any of the ascites. Another study with 2 ascites samples from 1 patient showed an increase in the P-glycoprotein, which has been associated with the pleiotropic drug resistance phenotype, in the second sample after treatment with cyclophcsphamide, doxorubicin

and cisplatinum (D.R. Bell et al, 1985). Wilson has also reported 2 ascites samples from 1 patient but with chemosensitivity data only from the primary culture of the first sample and a cell line derived from the second sample (A.P. Wilson, 1984) and sensitivity in the cell line also varied with passaging. Evidence for increased resistance of post-treatment samples (not all ovarian) has been provided in a study of 12 patients where multiple samples were available in the Hamburger/Salmon assay (S.E. Salmon et al, 1980). With ovarian adenocarcinoma it was feasible to collect a series of samples of the metastatic disease in the form of ascites fluid over the course of a patient's progress.

A second emphasis of this project was in concentrating on low levels of resistance since these are most probably of particular clinical relevance (as also emphasised by A. Rosowsky et al, 1985). The variation in drug sensitivity regarded as important is discussed at the end of Chapter 3 with my drug sensitivity results. Since patients are usually exposed to doses of antineoplastic drugs which approach the maximally tolerated dose which is limited by toxicity to normal tissues, a 3-fold increase in resistance of the tumour could not normally be matched by a 3-fold increase in dose of drug. This is consistent with the often seen initial response with later relapse and non-response to the same drug regimen. Thus the PE/01

and PE/04 resistant sublines were derived in cisplatinum concentrations up to 1uM which would normally be highly toxic to the parent cells, but no attempt was made to generate even more highly resistant sublines. Tumour heterogeneity with a range of drug sensitivity less than 10-fold (T. Tsuruo et al, 1981), for example a 5-fold range as observed by Yung in clonal glioma cell lines from a single tumour (W-K. A. Yung et al, 1982), would be very significant for therapy as discussed recently (A.J. Dembo, 1984; R.C.F. Leonard et al, 1985). These variations may be due to different microenvironments, especially for metastases (H. Rubin, 1985; S. Ahmad et al, 1986), as well as genetic differences (J.H. Goldie et al, 1984).

In looking at mechanisms of resistance an attempt was also made to stay at drug doses comparable with the biologically toxic dose (e.g. the LD_{50}) since too high doses of drug can lead to biochemical events irrelevant to the biological phenomenon of interest (F.N. Ghadially et al, 1981). This emphasis on low level resistance and biologically relevant doses created its own experimental problems. Many clues as to the possible biochemical mechanisms responsible for resistance have been obtained from highly resistant cells and the main possibilities for cisplatinum resistance have been investigated in Chapters 4,5 and 6. However as can be seen from the data, often a higher drug dose than that originally chosen needed to be

used, due to the sensitivity of the assays or to looking for differences between PE/01 and PE/04 which might be near to the variability seen in an assay. These problems were reason in part for deriving the PE/01 CisPt^R and PE/04 CisPt^R sublines with resistance to cisplatinum at somewhat higher levels so that possible differences between these lines could be observed. Nevertheless I believe these principles mentioned here are important if observations in tumour models are to have significance for clinical therapy. Our knowledge of mechanisms of drug action in various experimental systems now needs tc be translated to these more relevant models.

Until now the only clearly defined differences between PE/01 and PE/04 which may be relevant to drug resistance are the different interstrand crosslinking at the same dose of cisplatinum and the different level of glutathione and glutathione dependent enzymes, although reducing glutathione levels with BSO had little effect on cisplatinum toxicity. In PE/01 CisPt^R a further decrease in interstrand crosslinking was observed but no further increase in glutathione levels. The PE/04 CisPt^R subline was more difficult to derive than PE/01 CisPt^R and it is possible that a different mechanism of resistance was acquired. Certainly further investigations of the mechanisms of resistance are required in these 2 resistant sublines together with the 2 resistant lines (PE/04 and

PE/06) derived directly from ascites, particularly to see if there is a common mechanism of resistance or not. These experiments then need to be expanded to include cell lines (and ascites) from other patients in order to determine if general conclusions on cellular mechanisms important to resistance to cisplatinum can be made. Equally similar experiments with cross-resistant and non-cross-resistant platinum analogues would be useful.

During the course of this project efforts by others in this laboratory have focused on attempts to establish xenografts in neonatally thymectomised mice from the ovarian carcinoma cell lines and ascites. Early attempts using 10^7 cells injected subcutaneously appeared to give some tumours but these then regressed. Later efforts by Dr. Langdon in our laboratory has succeeded in establishing a subcutaneous xenograft from 10^8 injected cells of the PE/04 cell line which has progressed although growth is still slow. Some islets of tumour cells could also be seen from an intramuscular injection of 10^8 cells but no tumours grew from an intraperitoneal injection. Some work with PE/01, although with smaller numbers of cells, showed some subcutaneous growth although the tumours appeared to only grow to a certain extent and then stop. Clearly a very large tumour cell injection which is almost a solid tumour mass (10^8 cells in 0.1ml saline) is necessary to establish a xenograft. However it would

appear that both cell lines are tumorigenic. This experience is similar to that of others who found difficulty in establishing xenografts from ascites or cell suspensions (S. Kullander et al, 1978; P.J. Selby et al, 1980; G.G. Steel et al, 1983). Friedlander established xenografts from ascites or cell lines using a relatively high cell number (5×10^7 cells, M.L. Friedlander et al, 1985) and an intraperitoneal xenograft has also been reported (T.C. Hamilton et al, 1984). Clearly this sort of work is necessary to complement the work reported in my project and improve the overall model system used in this laboratory. An in vivo system is particularly useful in assessing drug combinations or modifiers of drug action. No attempt has been made to test drug combinations in vitro in this project since it is much more difficult to assess the significance of such in vitro results. Recent work at the I.C.R.F. in London has also established intraperitoneal ovarian tumours in nude mice from cell lines and ascites material with a good success rate (Dr. Bruce Ward, personal communication; I.C.R.F. Annual report 1985, section 26.10) and this may be a very appropriate model.

Other future studies of relevance for drug resistance include the phenotypic characterisation of cells (e.g. by a range of antibodies) to enable cells of different phenotype to be cultured together (but be still

identifiable) and their response to antineoplastic drugs then assessed, since subpopulation interactions may be very important for drug resistance (P.J. Tofilon et al, 1984). Another useful extension of the model in this regard would also be the development of spheroid cultures for looking at cell-cell interactions, relative hypoxia and drug penetration (T. Nederman, 1984; C. Erlichman et al, 1985) especially in those cell lines like PE/014 which naturally form domes and rounded cysts of cells in culture. Others have grown ovarian tumour cells on extracellular matrix instead of plastic (K. Crickard et al, 1986) and different drug sensitivity data have been reported for mouse mammary tumour lines in collagen gel cultures versus normal clonogenic assays (B.E. Miller et al, 1985).

The model presented here is now being broadened to test the other ovarian cancer samples and cell lines so that the observations made here can be generalised where possible. While in vitro sensitivity data, especially with cell lines, needs to compare a range of cell lines to attempt to reflect the clinical response of the tumour of origin (J.R.W. Masters et al, 1986), they can also suggest patterns of cross-resistance and possible common mechanisms of resistance. With related ascites cells or cell lines like the PE/01 family of lines the biochemical differences between them may be specifically related to

their different exposure to particular antineoplastic drugs without the addition of interpatient variability. In addition it is possible to make correlations between the drug sensitivity of cell lines (M. Albrecht et al, 1985) as well as primary samples (S.E. Salmon, 1984) with the clinical sensitivity of the tumour (ovarian carcinoma) from which they were derived.

7.2 Treatment of ovarian cancer - platinum drugs

Several recently published clinical studies have confirmed and extended the data on various combination chemotherapy regimens discussed in Chapter 1. The chemotherapy of ovarian cancer has been summarised in a number of recent reviews (C.J. Rodenburg et al, 1984; R.F. Ozols et al, 1984; M.H.N. Tattersall, 1985; G.S. Richardson et al, 1985; F.M. Muggia et al, 1985). Despite some recently reported reservations to the contrary (C.J. Williams et al, 1985; M.H.N. Tattersall et al, 1986) the consensus would appear to be that cisplatinum based combination regimens are at present the best choice for treatment of advanced ovarian cancer after primary surgery (R.F. Ozols, 1985; J.G. McVie, 1986). The CHAP-5 cisplatinum-containing regimen has been shown to be superior to the Hexa-CAF regimen (J.P. Neijt et al, 1984). In some instances cisplatinum also appears to be useful in second line treatment even in relapses after first line combination treatment including cisplatinum (V. Seltzer et

al, 1985; M. Bruzzone et al, 1986). High dose cisplatinum therapy (>100mg/m^2) has also received continued interest and shown responses even in patients resistant to standard doses (R.F. Ozols et al, 1985) The use of intraperitoneal therapy to administer a higher dose to the tumour has also been investigated further by a number of groups and has been recently reviewed (Gastrointestinal tumor study group intraperitoneal therapy workshop, 1985). Interest in other drugs (J.T. Thigpen et al, 1984) has continued to centre round the platinum analogues, particularly CBDCA (reviewed recently, S.K. Carter et al, 1985) but a number of analogues have received phase I and phase II trials (see Chapter 3 Discussion).

This clinical picture serves to confirm the emphasis of this project in focussing on cisplatinum as the drug of choice in ovarian cancer. Resistance to this drug is therefore critical. This appears to be most convincingly related to the amount of DNA lesions which are formed. The relative lack of cross resistance of CHIP and CBDCA to cisplatinum observed here (Chapter 3) is thus potentially very useful and the mechanisms of their action on ovarian carcinoma cells needs to be studied further. Clinically only an approximately 20-25% response rate has been observed for CBDCA in cisplatinum pretreated patients (R. Canetta et al, 1985; E. Wiltshaw, 1985) while preliminary data for CHIP suggest a similar picture (C. Sessa et al,

1986). Knox has suggested that cisplatinum and CBDCA are equally toxic if bound to the cell's DNA to the same extent (R.J. Knox et al, 1986) but CBDCA appears to be less reactive on a molar basis in forming DNA lesions like interstrand crosslinks (K.C. Micetich et al, 1985). In L1210 cells lack of cross-resistance to cisplatinum has also been reported for platinum complexes based on 1,2-diaminocyclohexane as the stable amine ligand (M.P. Hacker et al, 1985) as well as for some other analogues (W.C. Rose et al, 1984).

7.3 Drug resistance studies

Acquisition of drug resistance during the progression of many clinical tumours has been increasingly recognised as a critical problem to the continued improvement of cancer therapy and has been the subject of a number of recent reviews (G.A. Curt et al, 1984) extending to issues of journals (Cancer Treatment Reviews, 11:Suppl A, 1984; Cancer Surveys, 5(1), 1986) and books (B.W. Fox et al, 1984). Carter (S.K. Carter, 1984) and McGuire (W.L. McGuire et al, 1985) have discussed the present trends in the direction of research on the subject. Other reviews have emphasised drug disposition (J.G. McVie, 1984), the genetic nature of resistance (J.H. Goldie et al, 1984), the pleiotropic resistance phenotype and multidrug resistance (Cancer Treatment Reports, 67(10), 1983; J.H. Gerlach et al, 1986) gene amplification (R.T. Schimke,

1984; G.R. Stark, 1986) membrane alterations (D.W. Fry et al, 1986), DNA repair (M. Fox, 1984; A.L. Harris, 1985) collateral sensitivity (B.T. Hill, 1984) and tumour heterogeneity (D.L. Dexter et al, 1986). One recent new approach has been to isolate more sensitive mutants, rather than generate more resistant cells by exposing them to drug in vitro, with the idea that tumour resistance can almost by definition be equated with normal tissue sensitivity and therefore the nature of more sensitive cells is of most interest (C.N. Robson et al, 1985).

Resistance to cisplatinum at the cellular level has only recently become the subject of detailed attention (as discussed in Chapters 4,5 and 6) with most studies still seeking mainly to understand its mechanism of action (C.L. Litterst, 1984; J. Reedijk et al, 1985). However the number of papers presented at the recent meeting of the American Association for Cancer Research (Proc. Am. Assoc. Cancer Res., 27,1986) on this topic, with papers dealing with cisplatinum uptake (Nos. 1033,1038,1044,1069,1134 and 1155), cisplatinum and glutathione metabolism especially using BSO depletion of glutathione both in vitro and in vivo (Nos. 1139,1148,1155,1562 and 1665), intracellular metabolites (No. 1069), DNA adducts (Nos. 1033, 1046, 1132 and 1154), cross-resistance of analogues (Nos. 1133, 1134, 1138 and 1562) and rate of acquired resistance in vitro (No. 1549), illustrates the increased interest engendered

over the duration of this project and the subject has been recently reviewed (A. Eastman et al, 1986). One group at the NCI, Bethesda (T.C. Hamilton et al, 1984; T.C. Hamilton et al, 1985; Proc. Am. Assoc. Cancer Res. 27, 1986 Abstract Nos. 1046,1132 and 1562) and another at the University of California, San Diego (P.A. Andrews et al, 1985; Proc. Am. Assoc. Cancer Res. 27, 1986 Abstract Nos. 1069 and 1148) have used ovarian cell lines to study the mechanisms of resistance to cisplatinum. It is becoming apparent that as with other drugs resistance can be acquired in various ways and at the level of membrane, cytoplasm and nucleus with the possibility that different factors can be involved together in the resistance of a particular cell. In this project it seems clear that the amount of DNA lesions formed by cisplatinum is important but whether resistance is due to decreased formation of DNA adducts (affected by cytoplasmic or nuclear events) or increased DNA repair is uncertain. Reduction of transport of drug into the cell or effects of glutathione metabolism did not seem to play a major role. While some workers have concentrated on one aspect of the drug´s mechanism of action, usually through the availability of a particular assay, this can often lead to uncertainty as to the overall picture. I attempted to study as many different factors as possible in a new and unusual model, although more work leading on from the results presented here is obviously necessary before definite conclusions

can be drawn. It will also then be important to determine which factors turn out to be most commonly involved in vivo in the resistance of the majority of patients' tumours. Multiple factors may be particularly important for low level resistance although this is disputed by some (E.P. Clark, 1986). In addition different factors may be important for different analogues.

7.4 The future - overcoming drug resistance?

Apart from the importance of drug disposition and the responses of the tumour at a cellular level discussed above, other factors may be important. Cell-cell interactions have been shown to affect the sensitivity of cells to BCNU in mixtures of sensitive and resistant subpopulations (P.J. Tofilon et al, 1984). Another study using subpopulations of a mouse mammary tumour has shown one subpopulation to affect the chemosensitivity (to cyclophosphamide or methotrexate) of another subpopulation even when the subpopulations were injected on opposite flanks of the mouse, or in vitro when grown on separate coverslips in one Petri dish (B.E. Miller et al, 1981). An extracellular factor which transmits the cell response of UV-irradiated cells to non-irradiated cells has also been reported (M. Schorpp et al, 1984). Poste has suggested (in relation to the metastatic phenotype) that in polyclonal populations the various clonal subpopulations interact with one another to stabilize

their relative proportions and cloning results in rapid emergence of variant subclones (G. Poste et al, 1981). This might suggest that caution should be exercised in cloning cell lines in vitro to show heterogeneity of drug sensitivity since one may simply be introducing new variability on top of the selection presence of growth in vitro in the first instance. While tumour heterogeneity (within a primary tumour or between it and metastases, T. Tsuruo et al, 1981) is recognised as an important component in the resistance of tumours to chemotherapy (reviewed in Sem. Oncol. 12(3), 1985) the interactions between clones or subpopulations as well as their individual characteristics will need to be considered in attempting to overcome the resistance. For example in the experiments of Miller mentioned above a resistant subpopulation was more sensitive in the presence of the sensitive subpopulation. Thus once the sensitive population is eliminated the resistant subpopulation may emerge causing increased difficulty in treating the tumour. This might then be analogous to the problem of latent metastases (M.F.A. Woodruff, 1975).

Many studies have reported the increased efficacy of drug combinations and the experimental use of agents such as BSO (discussed in Chapter 5) to modify drug response. The effect of modification of glutathione and thiol metabolism on responses to radiation and chemotherapy has been the

subject of intensive study as discussed recently and is clearly more complicated than originally thought with possible membrane effects and compartments of glutathione in the cell (J.M. Brown (ed), 1986). An obvious extension of this project would be to test various combinations particularly as the in vivo counterpart of the model is developed. Experimentally cisplatinum has been combined with a number of other antitumour drugs (J-P. Bergerat et al, 1979a) with synergism particularly observed with cytosine arabinoside (AraC) (J-P. Bergerat et al, 1981; H. Vadi et al, 1986). Sequence dependent synergism has also been reported with dichloromethotrexate (A.F. Sobrero et al, 1985), and Etoposide (VP-16) (G. Zupi et al, 1985). Interactions of platinum complexes with x-irradiation have been observed (E.B. Douple et al, 1980) including experiments in organotypic cultures (R. Beaupain et al, 1985). In addition the effects of a number of modifiers of cell metabolism on cisplatinum toxicity have been reported including natural nucleosides (B. Drewinko et al, 1985a), polyamine depleters (S.M. Oredsson et al, 1982), inhibitors of ribonucleoside diphosphate reductase like hydroxyurea (G.R. Gale et al, 1979), calmodulin antagonists (Y. Kikuchi et al, 1984), interferon (J. Carmichael et al, 1986) and caffeine an inhibitor of post-replication repair (J.J. Roberts et al, 1986a). All these modifications may allow greater or more effective doses of drugs to be administered either by protection of host

cells or greater tumour cell kill. Some combinations can have increased effectiveness apparently just due to pharmacokinetic changes as with the effect of misonidazole on nitrogen mustards' action in vivo (F.Y.F. Lee et al, 1986) and the amount of drug reaching tumour cells needs analysis in assessing the biological response (P. Workman, 1986).

One recent attempt to transpose these experimental results into the clinical situation has been that of Markman who treated patients mostly with ovarian cancer with intraperitoneal cisplatinum and AraC concomitantly with intravenous sodium thiosulphate (M. Markman et al, 1985). The intraperitoneal route allows high doses of cisplatinum and AraC to be administered to the tumour while also potentially exploiting the synergism between the two drugs, and the intravenous sodium thiosulphate helps to prevent systemic toxicity due to cisplatinum. Responses in previously refractory patients were observed. Conte has attempted to induce (with iphosphamide), and monitor, recruitment of ovarian ascites cells into S phase so as to schedule antimetabolite treatment at the appropriate time although without observing tumour responses as yet in refractory patients (P.F. Conte et al, 1984). There are also some reports of sequential or alternating therapy in ovarian cancer based on the Goldie-Coldman hypothesis (A.J. Dembo, 1984) but not so far with results improved

over standard therapy (M.W. Pasmantier et al, 1985; F. Lawton et al, 1985).

Obviously new drugs are needed to improve present therapy and, while better use of present drugs and introduction of new analogues will be helpful, entirely new types of drugs and therapy can be envisaged. The search for agents to induce cell differentiation (A. Bloch, 1984) has recently become an active area of research. Where the cancer has reached an advanced stage with perhaps a highly heterogenous population with multiple resistance the goal must be to use different therapeutic approaches together, as DeVita (1983) has said, "to give the cancer cell a series of impossible choices".

This project has developed a model for investigating drug resistance in ovarian cancer in vitro and examined resistance and mechanisms of resistance to cisplatinum together with cross-resistance to analogues of clinical interest. It is hoped that this research and the ideas put forward as a result of it may contribute in the future to improvements in the outcome of treatment for ovarian cancer.

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APPENDIX

CELLULAR HETEROGENEITY AND DRUG RESISTANCE IN TWO OVARIAN ADENOCARCINOMA CELL LINES DERIVED FROM A SINGLE PATIENT

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Abbreviations: HSR: homogeneous staining region
PBS: Dulbecco's phosphate buffered saline
PAP: Papanicolaou
PAS-diastase: periodic acid Schiff

Running Title: Drug resistance in ovarian cancer

ABSTRACT

Two ovarian cell lines have been derived from the ascites of a patient before and after the onset of resistance to chemotherapy involving cis-platinum, chlorambucil and 5-fluorouracil. Characterization of these lines shows them to have various features in common and some significant differences. Cytologically the lines cannot be distinguished and they both contain high concentrations of oestrogen receptor. However they do differ with respect to their growth characteristics, karyotype, glutathione content and sensitivity to cis-platinum. The karyotypes of the two lines show several marker chromosomes in common but the resistant line contained a chromosome 8 and 17 absent in the earlier sensitive line. This suggests a clonal origin with subsequent divergence to a heterogeneous population.

INTRODUCTION

Ovarian adenocarcinoma is a disease responsive to a variety of chemotherapeutic agents (Rodenberg & Cleton, 1984). However the remissions which result from treatment are often short, and following relapse the patients are usually resistant to subsequent chemotherapy (Rodenberg & Cleton, 1984). There are numerous possible explanations why tumours are refractory to treatment (Hill, 1982). Firstly, there may be alterations in drug disposition not related to the tumour cells themselves. Secondly, there is the possibility that the tumour cells when exposed to cytotoxic compounds acquire resistance to them. The phenomenon of acquired resistance has been extensively studied and is well characterized for many cell culture model systems (Schimke, 1984; Louie et al. 1985). In the majority of reports, resistant cells have been produced in vitro by selection, i.e. by growing the cell lines in the presence of the drug under investigation (Fojo et al. 1985). In view of the relative difficulty in obtaining tissue samples and transferring them

successfully into culture (Bertoncello et al. 1982), there are still only a few reports which unequivocally indicate that acquired drug resistance is an important factor in the failure of chemotherapy (Bell et al. 1985). A third explanation for resistance is the presence of minor cell populations within the original tumour which are intrinsically more resistant to chemotherapy and that following initial treatment these cells become the major population within the tumour. This represents the basis of the Goldie-Coldman model (Goldie & Coldman, 1979) and is supported by many reports of tumour cell heterogeneity (Heppner, 1984).

In order to investigate these possibilities, we have obtained ascites samples from a patient before and after the onset of clinical drug resistance. Two cell lines have been established from these samples, characterized, and tested for drug sensitivity.

MATERIALS & METHODS

Source of Ascites Samples and Cell Lines

The patient who was diagnosed as having ovarian adenocarcinoma in April 1980 was treated with seven courses of 5-fluorouracil (500 mg/m^2 I.V.), cis-platinum (30 mg/m^2 I.V.) and chlorambucil (0.1 mg/kg oral) over a period of 7 months. A complete response - determined by second-look laparotomy - was obtained and no further treatment was given until relapse 14 months later. A sample of ascitic fluid was obtained at this time and the cells were isolated and put into tissue culture (cell line PE01). The patient then received a further six courses of treatment (February-August 1982) as described above and responded well initially but relapsed in November 1982. The patient was then treated with a higher dose of cis-platinum (100 mg/m^2) but did not respond. A cell line was derived from a second ascites sample (PE04) taken in December 1982. The patient died in February 1983.

The cell lines were derived in the following manner. The freshly obtained ascites samples were

mixed with 100 units of preservative-free heparin and the cells sedimented by centrifugation. After the cells had been washed twice with phosphate-buffered saline (PBS), red cells were removed using a Ficoll Hypaque gradient. Tumour cells at the interface were aspirated off, washed twice with PBS and checked for viability by nigrosin staining. Aliquots of 10^5 cells per ml were then cultured at 37°, 100% humidity and 5% CO_2 in RPMI 1640 + 10% foetal calf serum (v/v) with added insulin (2.5 µg/ml), streptomycin (100 µg/ml), penicillin (100 IU/ml) and 3-[N-morpholino]propanesulphonic acid (12.5 mM). PE01 and PE04 were subcultured after 21 and 4 weeks respectively. Confluent cultures were passaged by 1:5 or 1:10 splits. Periodic assays for mycoplasma were negative.

Cytology

Cells at passage 1 and 79 of PE01 and 2 and 48 of PE04 were harvested and grown on sterile 22 x 22 mm coverslips in 35 mm wells (6 well plates, Sterilin, UK). When good, even growth was obtained the cells were fixed and stained with PAP

to reveal gross morphology or with PAS-diastase to stain for mucin.

Karyotyping

Chromosome preparations were made from the patient's peripheral blood as well as the cell lines PEO1 and PEO4 at various passages in culture. These preparations were stained with spermidine bis-acridine by the Q-band technique of Van de Sande et al (1979).

Drug Sensitivity Assay

Drug sensitivity assays were carried out at the same time for the 2 cell lines. Cells were at passages 77 to 82 and 46 to 51 for PEO1 and PEO4 respectively.

a) Clonogenic Assay in Soft Agar

The in vitro soft agar assay of Courtenay et al (1978) was used. A single cell preparation was obtained by vigorous aspiration and passage through a 19 gauge needle. Viability was checked with nigrosin stain. 5×10^3 and 1×10^3 cells/ml in a test tube for PEO1 and PEO4 respectively were incubated with drug in the agar for 21 days with 1 ml of fresh medium added on days 7 and 14.

Colonies of greater than 50 cells were scored visually.

b) Clonogenic Assay on Plastic

Assay conditions were modified from those described by Parsons & Brown (1979) to suit these ovarian cell lines. Cells were plated out in 35 mm wells (6 well Linbro plates, Flow Laboratories, UK) at 2 x 10^3 and 10^3 cells/well for PEO1 and PEO4 respectively in medium containing 1mM pyruvate to give 100 to 200 colonies in control wells. After 2 days, when cells had firmly attached but had not yet divided, drugs were added to wells in triplicate and left on for 3 days. Medium was changed every 2 to 3 days. After 12 to 14 days colonies were counted visually.

Colony Forming Efficiency and Doubling Time

The colony forming efficiency of the lines in agar and on plastic was determined in the Courtenay assay and the assay on plastic as described. Doubling time was determined by plating at 5 x 10^3 cells per well in a 24 well plate (NUNC, Gibco Ltd) and harvesting rows of 4

wells at the same time each day for 12 days. Harvested cells were counted by Coulter Counter.

Glutathione & Glutathione Transferase Assays

Glutathione levels were determined by HPLC following derivitization with monobromobimane (Gaetjens, 1984). Glutathione transferase activity using 1-chloro-2,4-dinitrobenzene as substrate was determined by the method of Habig et al (1974). Cells used were in log phase and the values were expressed per 10^6 viable cells.

Hormone Receptor Assays

Oestrogen and progestogen receptor assays were carried out on confluent cell cultures maintained for 6 to 10 days in steroid stripped medium (Hawkins et al. 1981; Hamilton et al. 1983).

DNA Content

DNA content was measured on a fluorescent-activated cell sorter using human lymphocytes as an internal diploid marker. Only a single G_1 peak was observed.

RESULTS

i. Cytology

Examination of the ascites samples and the cell lines derived from them is shown in Figure 1. In contrast to the original ascites sample taken in April 1980, the two later samples from which cell lines PEO1 and PEO4 were derived were largely devoid of both macrophages and lymphocytes. The ascitic fluids contained groups of cells as well as single cells of adenocarcinomatous type, characterized by large nuclei eccentrically situated in the cytoplasm and occasional signet ring cells due to the presence of a large vacuole containing mucopolysaccharide. The latter property is consistent with ovarian metastases. Cytological examination of the cell lines showed them to be very similar to the cells of the ascitic fluid (Figures 1d & 1e). Both cell lines had large oval nuclei and prominent, sometimes multiple, nucleoli, contained signet ring cells and were mucin secreting. No cytological difference between the cell lines or between different passages of the cell lines was apparent

even at the ultrastructural level by electron microscopy (results not shown).

ii. Drug Sensitivity

The sensitivity of PEO1 and PEO4 to the cytotoxic drugs used clinically, i.e. cis-platinum, chlorambucil and 5-fluorouracil, tested using clonogenic assays in agar and on plastic is shown in Charts 1, 2 & 3. Two different assays were used to give us greater confidence in the significance of the drug sensitivity differences between the 2 cell lines. A 3-fold difference in the sensitivities of the lines to cis-platinum was measured using either assay. Both assays gave similar results, the LD_{50} values being $8.0 \times 10^{-8}M$ and $2.7 \times 10^{-7}M$ in the agar assay and $6.4 \times 10^{-8}M$ and $2.0 \times 10^{-7}M$ in the assay on plastic for PEO1 and PEO4 respectively. For chlorambucil the sensitivity difference was 3-fold in the plastic assay and only 1.3-fold in the assay on agar. In the case of 5-fluorouracil, no differences between the cell lines were measured by either assay. Although there was more variation between experiments in measuring sensitivity to 5-fluorouracil,

probably due to variation in the nucleosides in serum batches, no differences between the cell lines within an experiment were observed. Both cell lines appeared more sensitive to 5-fluorouracil in the agar assay.

iii. Cell Line Characteristics

A summary of the general properties of the two cell lines are shown in Table 1 and Figure 2. PEO4 had a higher colony efficiency. Both glutathione and glutathione transferase activity, components central to the deactivation of alkylating agents (Mitchell et al. 1982), were approximately 2-fold higher in PEO4 than in PEO1. Both cell lines had high levels of oestrogen receptor, the value for PEO4 being slightly higher than that of PEO1, and undetectable levels of progestogen receptors after 10 days culture in steroid stripped medium (Hamilton et al. 1983).

iv. Karyotype

Karyotypes of the cell lines and peripheral blood are shown in Figure 3.

The cells from the peripheral blood showed a normal 46,XX karyotype even after the chemo-

therapy, whereas the cell lines from the ascites were hypodiploid. However their DNA content was slightly greater than that of normal diploid human lymphocytes. The modal chromosome numbers from different spreads varied between 39 and 42. Examples of the chromosome complement of PEO1 and PEO4 with 41 chromosomes are illustrated (Figures 3b & 3c). Figure 3a is a Q-banded karyotype made from a normal peripheral blood cell taken from the patient 4 weeks before she died. The cultured ascites cells, in addition to being hypodiploid, contained many abnormal chromosomes. The chromosomes that are considered to be normal are marked with a white square. PEO1 has only 20 such chromosomes. None of the cells analysed contained a normal 8 or 17 chromosome or any material that could be considered as coming from either of these chromosomes although this cannot be excluded because there are so many rearranged chromosomes. PEO1 had 12 consistent abnormalities which were: both 3s inv(3)(p-q+), 5p-, 9p+, 11p+, both 13s 13q- and 13q+, 22q+ and 4 chromosomes of unknown origin. The number and appearance of the other

abnormal chromosomes varied. Of the 4 chromosomes of unknown origin 2 marker chromosomes had a similar appearance - two prominent bands on the distal long arm but the pericentric chromatin was very pale, uniformly staining and reminiscent of homogeneous staining regions (HSR). These regions were not C-band positive. The other two chromosomes appeared to be isochromosomes being metacentric with a very similar banding pattern each side of the centromere, one being half the size of the other.

In contrast, PEO4 had an apparently normal member of each chromosome pair including a chromosome 8 and 17 but excepting an X and 13 chromosome. However, the 13q- chromosome now has material translocated to the brilliant p11 which could make this chromosome complete although rearranged. Of the consistent abnormalities seen in PEO1, the following are seen in PEO4: inv(3)(p-q+) with the brilliant Q-band, 5p-, 9p+, 11p+, 13q+, 22q+, one of the marker chromosomes with the possible HSR and in many cells the smaller of the two isochromosomes was seen. PEO4

had acquired a ring chromosome which was approximately the size of a D group chromosome, and also other rearranged chromosomes.

The 'normal' 22 in both PEO1 and PEO4 has lost the intense satellites. Deletion of satellites or the whole of an acrocentric short arm is often found as a constitutional 'abnormality' in normal individuals.

DISCUSSION

Two ovarian adenocarcinoma cell lines have been established from ascites obtained before and after the onset of resistance to chemotherapy. A marked difference was observed in the sensitivity of these lines to cis-platinum, one of the chemotherapeutic agents used for treating the patient. Although the difference in sensitivity of these lines to cis-platinum was only 3-fold, we believe that changes in drug sensitivity of this order of magnitude would be important clinically (Ozols et al. 1984; Hamilton et al. 1984). No change in drug sensitivity with passaging in vitro was observed. Whilst not the only possible expla-

nation, our in vitro data suggest that the tumour cell population or subpopulations changed in the patient during the drug treatment and the difference in drug sensitivity could explain the resistance of this patient to further treatment. In support of such in vivo change is the fact that the PE04 cell line was derived more rapidly and with much less difficulty than the PE01 line. The LD_{50} levels reported here are at the lower end of the range for cis-platinum toxicity values (0.03-3 μM) reported in the literature for ovarian (Holzel et al. 1985; Buick et al. 1985; Van Putten et al. 1986) and other human cell lines (B.T. Hill et al. 1984) and below that reported for normal human bone marrow cells (0.9 μM) (Umbach et al. 1985).

Although the cell lines were cytologically similar, there were significant differences in their karyotypes. Chromosome abnormalities found in the tumour cells of other patients with ovarian carcinoma have also been bizarre, and inevitably some of the rearranged chromosomes in these tumours have involved the same chromosomes as in the tumour cells from the present case (Hamilton

et al. 1983; Freedman et al. 1978; S.M. Hill et al. 1984; Kakati et al. 1975; Van der Reit-Fox et al. 1979; Woods et al. 1979). However, we did not find the (6;14) translocation which it is suggested may characterise this carcinoma (Trent & Salmon, 1981), although we did observe a 3p⁻marker similar to that observed by others (Panani & Ferti-Passantonopoulou, 1985; Mackillop et al. 1983).

No double minutes were found in these cells. The appearance of two marker chromosomes in PEO1 was consistent with them carrying homogeneous staining regions (HSRs). HSRs have been, but need not necessarily be, associated with the acquisition of drug resistance (Schimke, 1984). It is interesting that the number of HSRs was reduced in PEO4. PEO4 appears to contain more 'normal' chromosomes than the earlier PEO1 culture. Since both cell lines have several rearranged chromosomes in common and were cytogenetically very heterogeneous, it could be argued that the two cell lines probably arose from a common ancestor, both diverged and co-existed in the body, but that

PEO1 was more susceptible to the initial drug treatment and was overgrown by PEO4. It is very unlikely that PEO4 evolved from PEO1 as it would be almost impossible to regain a normal 8 and 17 having once lost them. The two cell lines therefore represent a model for the study of human tumour cell heterogeneity. There was no evidence that the karyotype or properties of the cells changed during passaging in vitro.

Variation in sensitivity to cis-platinum due to tumour cell heterogeneity has been observed in clonal human glioma lines isolated from a single untreated tumour (Yung et al. 1982). This sort of heterogeneity has been observed by others (Tsuruo & Fidler, 1981) and has also been found in primary ovarian cancer samples (Siracky, 1979). Our results which suggest PEO4 is not a lineal descendent of PEO1 would support the importance and relevance of sub-population heterogeneity in drug resistance.

PEO1 and PEO4 contained extremely high levels of oestrogen receptor but undetectable levels of progestogen receptors. Whether progestogen

receptors could be induced by oestrogen, specifically excluded from the assay here, is as yet unknown. Oestrogen and progestogen receptor positive ovarian tumours and cell lines have been reported (Hamilton et al. 1983; Lazo et al. 1984) but the levels of oestrogen receptor detected here are very high compared with most reports (eg mean of 18 and maximum of 163 fmol receptor/mg cytosolic protein in 56 ovarian tumour samples reported by Lazo et al, [1984]).

Two assays have been used in this study to investigate drug sensitivity to increase our confidence in differences in sensitivity measured. These were a relatively simple assay involving colony counting of attached colonies in 6-well plates and the more complex assay involving colony growth in soft agar. The comparative sensitivity of the cell lines determined with these assays was similar although some differences were apparent. The reason why chlorambucil gave a larger sensitivity difference between PEO1 and PEO4 using the assay on plastic is unclear but preliminary experiments suggest it may be related to the

increased oxygen tension (20% O_2 vs. 5% O_2). The growth conditions in agar are clearly different to those in monolayer on plastic. In particular anchorage dependence (Stephens et al. 1980), oxygen tension (Gupta & Krishnan, 1982) and the addition of rat red blood cells in the Courtenay assay (Endresen et al. 1985) have all been shown to affect chemosensitivity measurements for some cells. Also the metabolic state of the cells when treated in suspension after trypsinisation, or after attachment has been shown to be important (Twentyman, 1979).

These conditions may also explain the difference in sensitivity to 5-fluorouracil between the 2 assays although other factors such as the presence of thymidine (Engelbrecht et al. 1984) in the Hams F-12 medium (and not in RPMI-1640) used in the Courtenay assay or the length of time (3 days vs. 21 days) of drug exposure (Sobrero & Bertino, 1983) may also influence the results. The data here would suggest that great care should be taken in relating in vitro assay results back to a patient since different assays

can give different results, as here with chlorambucil.

It is interesting to note the difference in glutathione levels in our cell lines. The role of glutathione in the protection of cells from cis-platinum is not clear. However the role of this peptide in the detoxification of alkylating agents in general is well established (Mitchell et al. 1982). There is also some evidence which suggests that thiol-containing compounds can increase the survivals of cis-platinum treated mice as well as reduce both the kidney and gastro-intestinal tract toxicity of cis-platinum (Allan et al. 1986; Borch & Pleasants, 1979). The potential importance of intracellular thiols is exemplified by a recent report by Green et al (1984) who demonstrated that an ovarian cell line resistant to melphalan was made sensitive to this compound by suppressing glutathione biosynthesis. In our cell lines the glutathione levels are similar to those of other ovarian cell lines as reported by Louie et al (1985). Other workers have suggested that glutathione metabolism may be

more important for melphalan and chlorambucil toxicity than for cis-platinum (Andrews et al. 1985). However, the higher thiol level in PEO4 may be a contributing factor to its increased resistance demonstrated in the present study.

The overall sensitivity or relative resistance of ovarian cancer to cis-platinum is multifactorial. Differences in thiol levels are just one of these factors. Tumour heterogeneity, demonstrated here cytogenetically, is also clearly important. Compared with the relative restriction of working with fresh ascites we believe that the cell lines described in this paper constitute an appropriate and useful model for the further study of mechanisms of drug resistance in this important human tumour.

Acknowledgements

The authors would like to thank Dr R A Hawkins, Department of Clinical Surgery, University of Edinburgh for carrying out the hormone receptor assays.

TABLE 1

CELL LINE CHARACTERISTICS

aracteristics	PEO1	PEO4
ibling time (hrs)	53	59
in production	+	+
ony forming efficiency astic)	3.9 ± 2.3	8.1 ± 3.8
ony forming efficiency ar)	3.9 ± 2.5	18.3 ± 10.4
content	1.1x diploid	1.1x diploid
al chromosome no.	41	41
ormal chromosomes	+	+
trogen receptor mol/mg protein)	145 ± 34	203 ± 23
gestogen receptor mol/mg protein)	Not detectable	Not detectable

1 and PEO4 are two cell lines derived from the same patient with carcinoma of ry before and after developing clinical resistance to cis-platinum. For ails see text.

FIGURE LEGENDS

FIGURE 1:

Cytology of Ascites and Cell Lines Samples

Samples were prepared and stained with Papanicalaou stain as described in the MATERIALS & METHODS.

a) Ascites cells April 1980

b) Ascites cells February 1982

c) Ascites cells December 1982

d) Cell line PEO1 passage 79

e) Cell line PEO4 passage 48

FIGURE 2:

Glutathione and Glutathione-S-transferase Activity in Cell Lines PEO1 and PEO4

Glutathione and glutathione-S-transferase activity was measured as described in materials and methods. Hatched boxes represent glutathione content and open boxes glutathione-S-transferase activity. Values were means of three separate experiments carried out in triplicate, bars represent the standard error.

FIGURE 3:

Karyotypes of Peripheral Lymphocytes, and Cell Lines PEO1 and PEO4

a) A Q-band karyotype of a normal cell from a peripheral blood culture, showing the Q-band polymorphisms.

b) Karyotype of a cell from PEO1. The chromosomes marked with a white square are considered to be normal. The abnormal chromosomes which are arrowed are also found in PEO4.

c) Karyotype of a cell from PEO4. Symbols are as above for figure b. The chromosomes marked + are either normal chromosomes or consistent chromosome abnormalities not found in PEO1.

CHART 1

Drug Sensitivity for Cell Lines PEO1 and PEO4 Using Cis-Platinum

a) Clonogenic assay in soft agar according to Courtenay et al (1978). Points, means of 1-2 experiments PEO1 and 2-6 experiments PEO4, bars represent the standard error.

b) Clonogenic assay on plastic. Assay conditions as described in the MATERIALS AND METHODS section. Points, means of 2-4 experiments, bars represent the standard error.

CHART 2

Drug Sensitivity for Cell Lines PEO1 and PEO4 using Chlorambucil

Legend as in Chart 1. Points, means of 2-5 experiments, bars represent the standard error.

CHART 3

Drug Sensitivity for Cell Lines PEO1 and PEO4 Using 5-Fluorouracil

Legend as in Chart 1. A typical experiment is shown. Points, means of 5 replicates (agar) or triplicates (plastic), bars represent the standard error.

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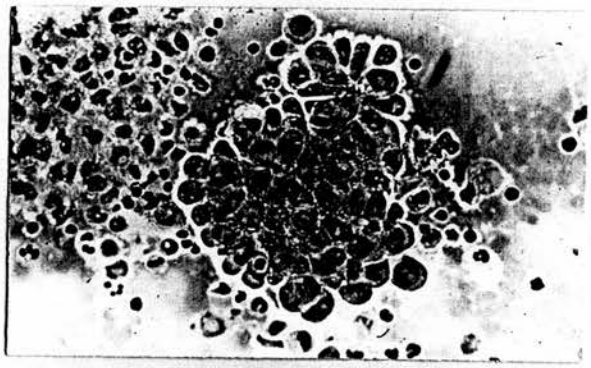
Figure 1

1a.



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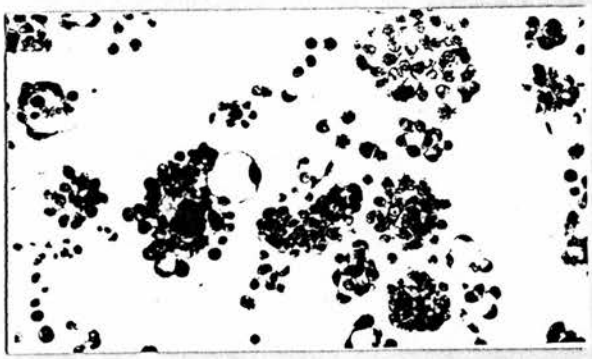

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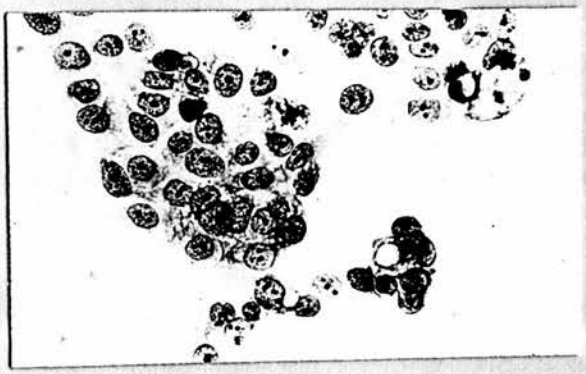

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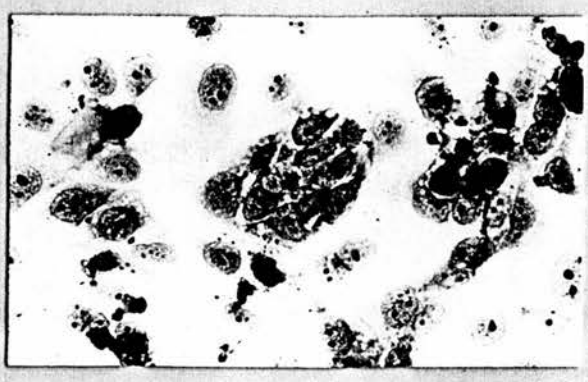


Figure 2

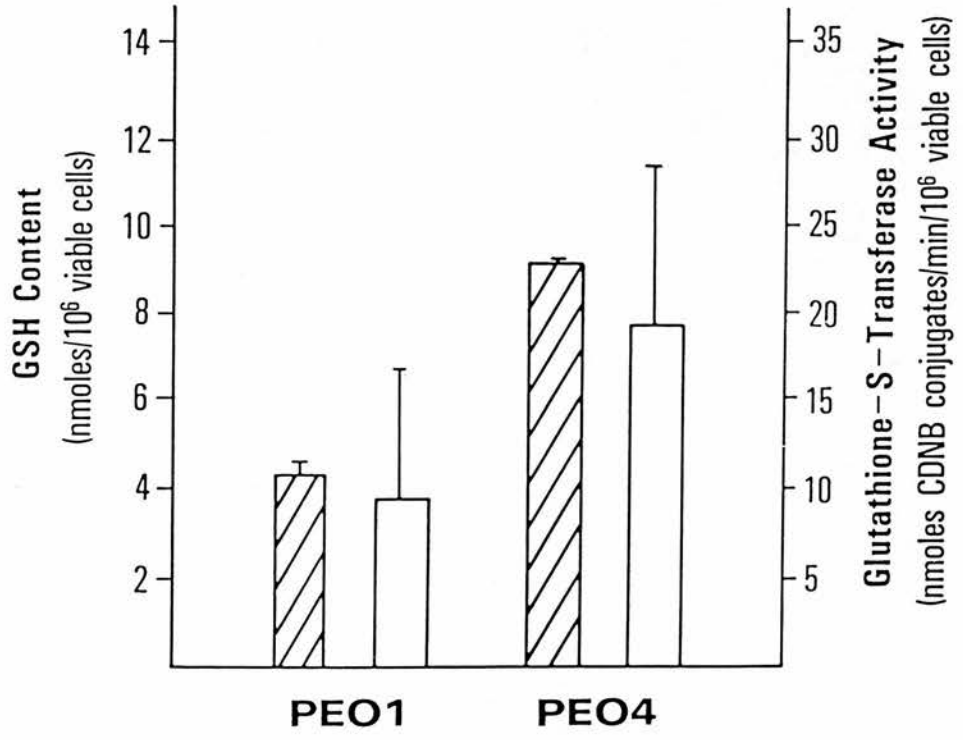
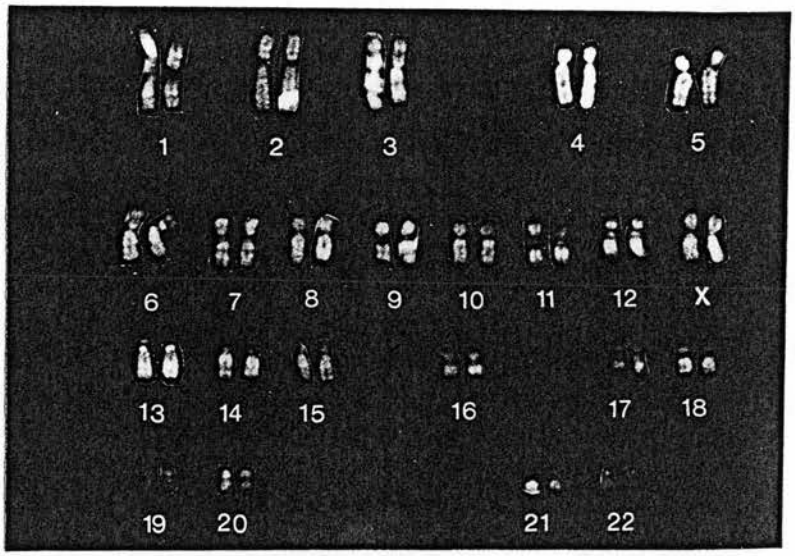

GSH Content
(nmoles/10⁶ viable cells)
14
12
10
8
6
4
2
35
30
25
20
15
10
5
Glutathione-S-Transferase Activity
(nmoles CDNB conjugates/min/10⁶ viable cells)
PEO1
PEO4

Figure 3

3a.



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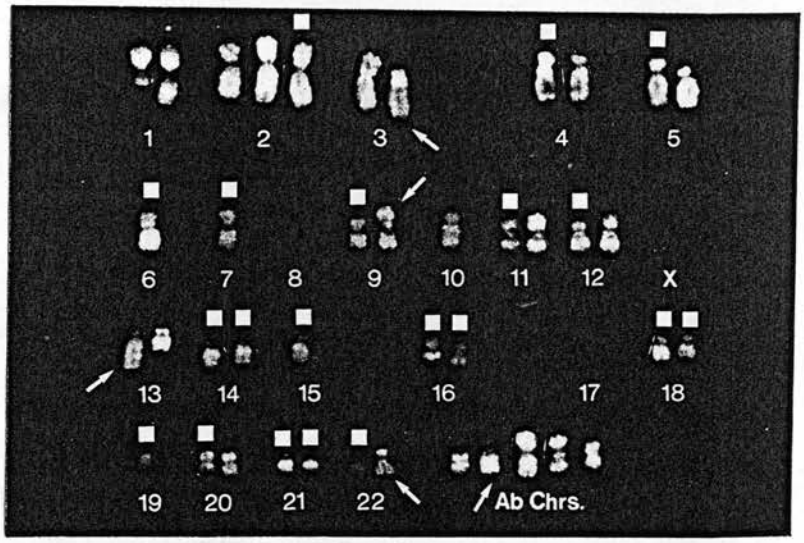

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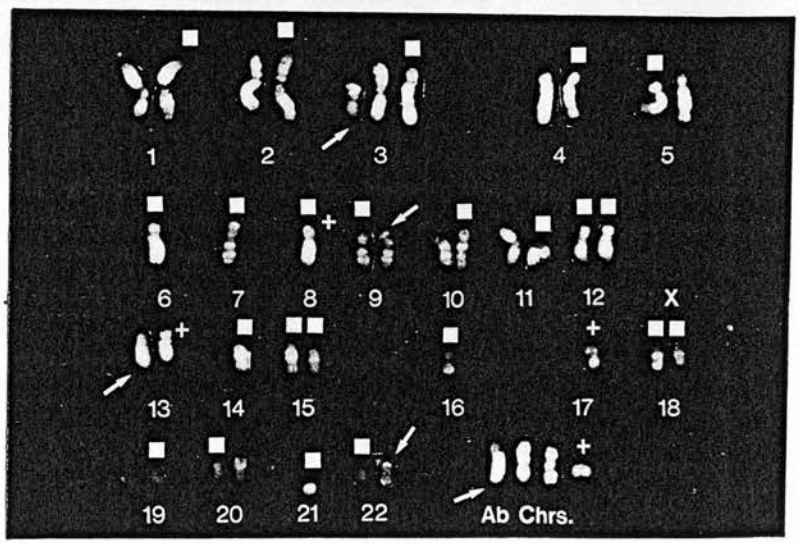
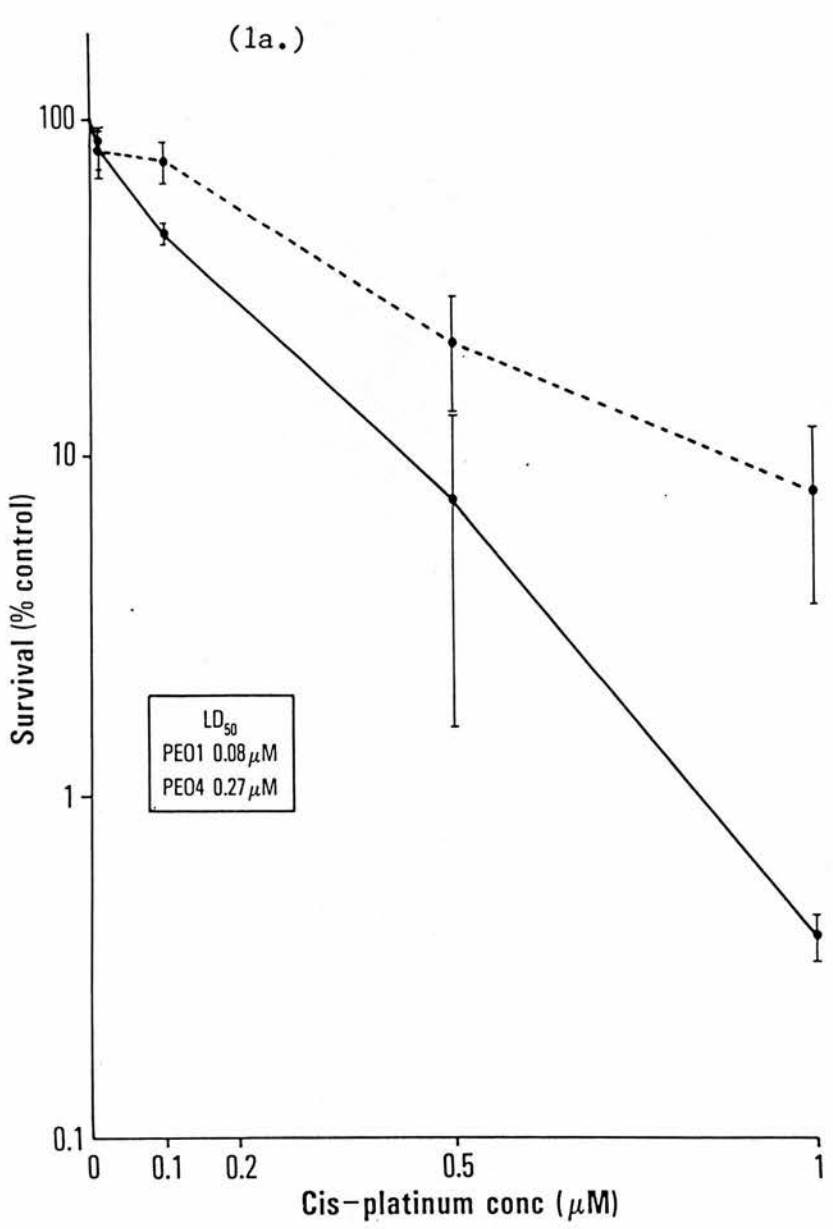


Chart 1


(1a.)
100
10
1
0.1
Survival (% control)
LD50
PEO1 0.08 μM
PEO4 0.27 μM
0
0.1
0.2
0.5
1
Cis-platinum conc (μM)

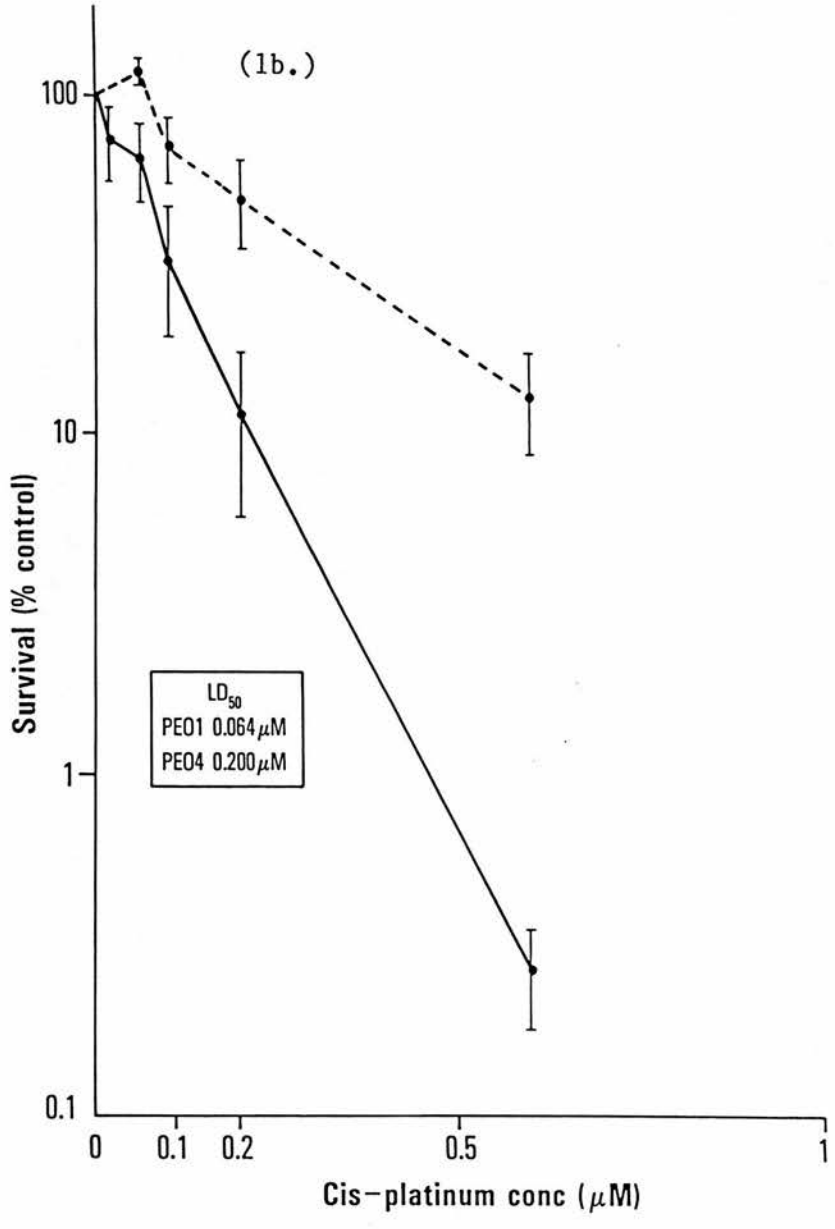
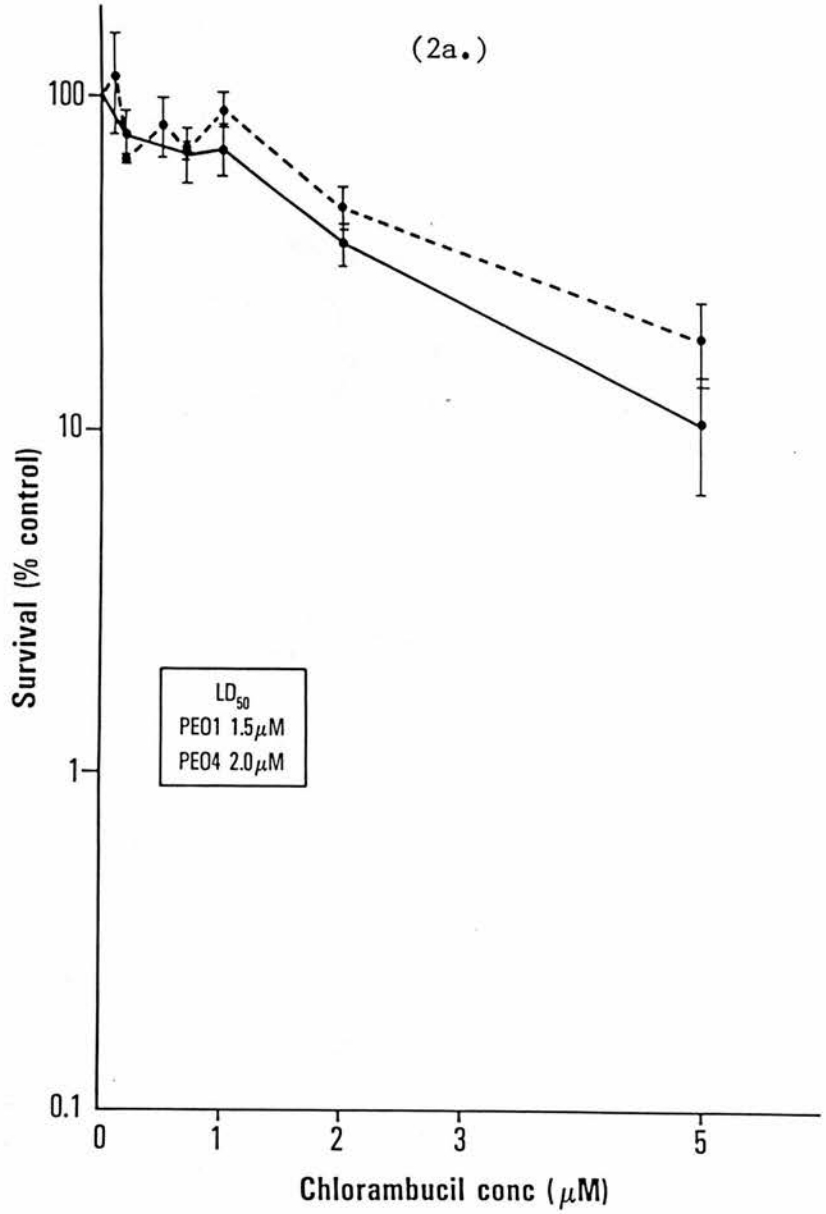

(1b.)
100
10
1
0.1
Survival (% control)
LD50
PEO1 0.064 μM
PEO4 0.200 μM
0
0.1
0.2
0.5
1
Cis-platinum conc (μM)

Chart 2


(2a.)
100
10
1
0.1
Survival (% control)
LD50
PEO1 1.5μM
PEO4 2.0μM
0
1
2
3
5
Chlorambucil conc (μM)

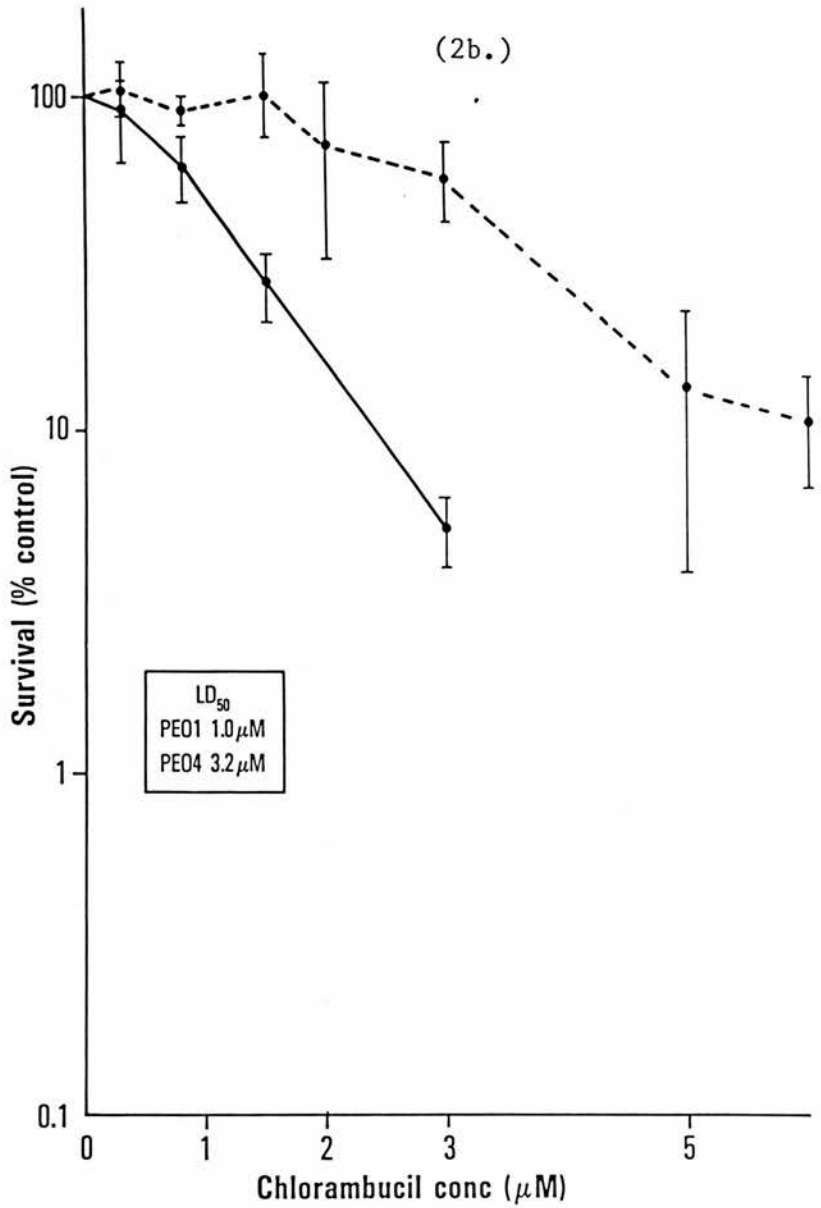
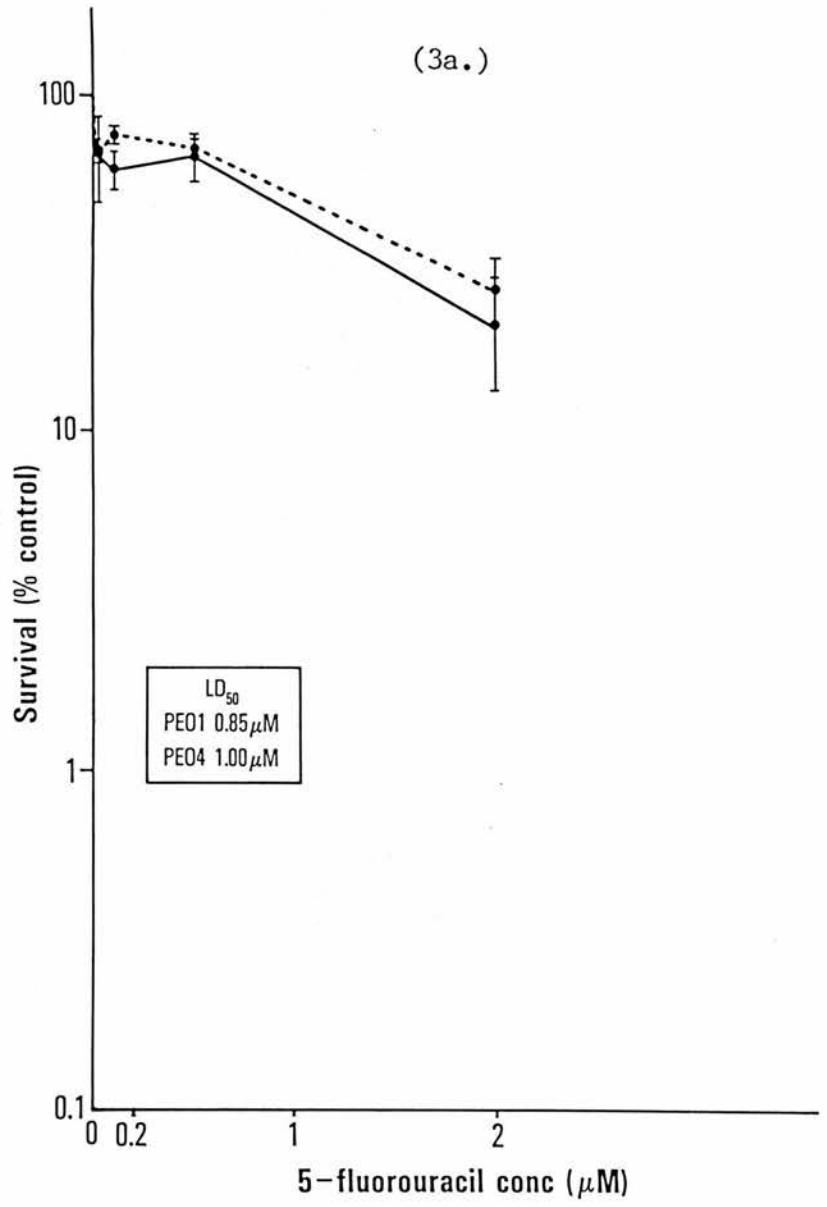
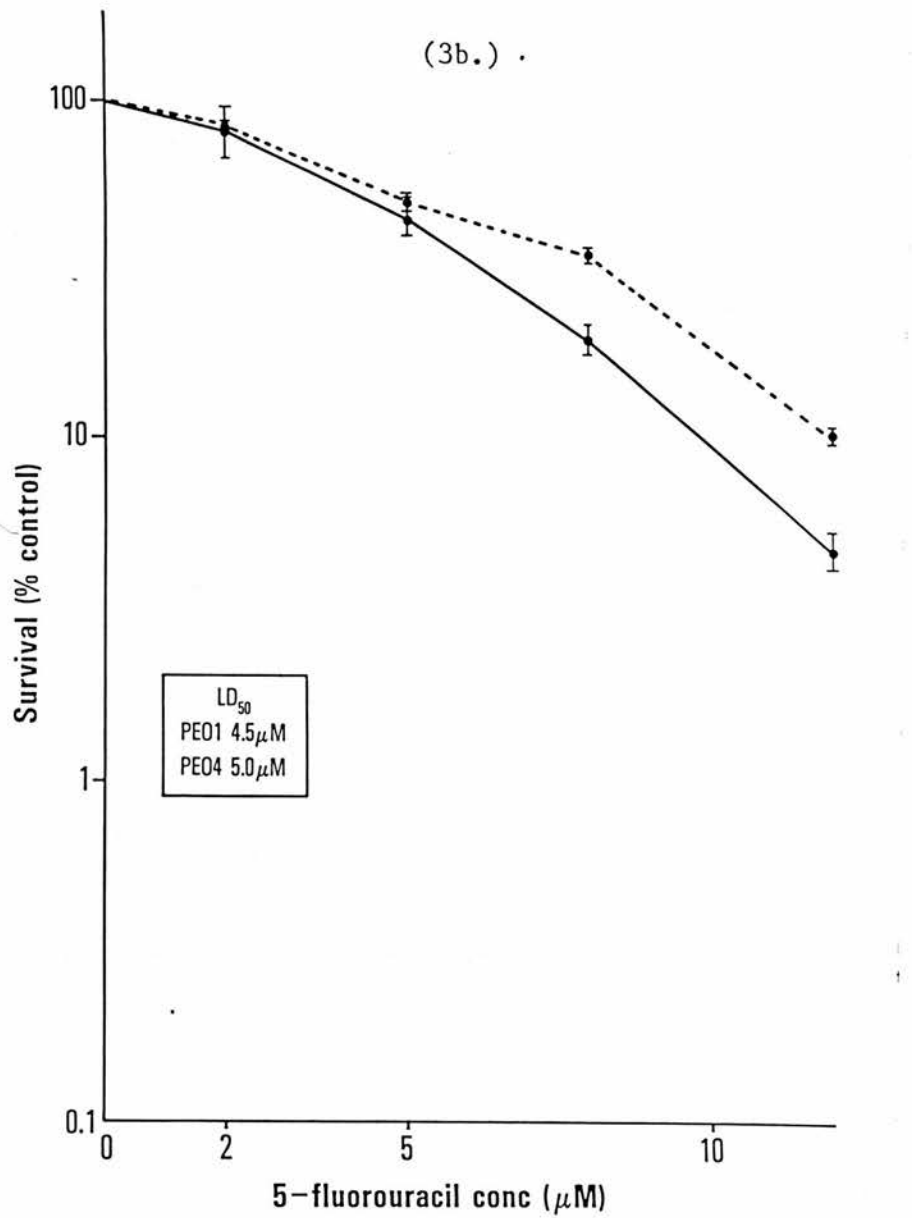

(2b.)
100
10
1
0.1
Survival (% control)
LD50
PEO1 1.0μM
PEO4 3.2μM
0
1
2
3
5
Chlorambucil conc (μM)

Chart 3


(3a.)
100
10
1
0.1
Survival (% control)
LD50
PEO1 0.85μM
PEO4 1.00μM
0
0.2
1
2
5-fluorouracil conc (μM)


(3b.)
100
10
1
0.1
Survival (% control)
LD50
PEO1 4.5μM
PEO4 5.0μM
0
2
5
10
5-fluorouracil conc (μM)